# Multiple Specificities in the Repertoire of a Melanoma Patient's Cytolytic T Lymphocytes Directed against Tumor Antigen MAGE-1.A1

By Pedro Romero,* Christophe Pannetier,‡ Jean Herman,§
C. Victor Jongeneel,* Jean-Charles Cerottini,* and Pierre G. Coulie§

*From the *Ludwig Institute for Cancer Research, Lausanne Branch, University of Lausanne, CH-1066 Epalinges, Switzerland; ‡Unit of Molecular Biology of the Gene, Unité 277 Institut National de la Sante et de la Recherche Medicale, Pasteur Institute, 75724 Paris Cédex 15, France; and §Ludwig Institute for Cancer Research, Brussels Branch, B1200 Brussels, Belgium*

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## Summary

Peptide MAGE-1.A1 is a nonamer derived from protein MAGE-1 that can associate with the HLA-A1 molecule. It was shown previously to be recognized by an antitumor cytolytic T lymphocyte (CTL) clone derived from the blood of melanoma patient MZ2. We derived two other anti-MAGE-1.A1 CTL clones from different blood samples of the same patient and compared the fine specificity of recognition of the three CTL by testing them on variant MAGE-1.A1 peptides incorporating different amino acid substitutions. The epitopes recognized by the CTL proved to be different. While modifications of residues at positions 5, 6, or 7 in the antigenic peptide affected recognition by the three CTL, each of the modifications of residues at positions 1, 4, or 8 affected recognition by one CTL only. The sequences of both the α and β chains of the T cell antigen receptor of the three CTL were completely different. The results indicate a long-lasting diversity in terms of fine specificity and of T cell antigen receptor structure in the repertoire of antitumor CTL derived from the blood of a melanoma patient and directed against a defined tumor antigen.

When blood lymphocytes of melanoma patient MZ2 are stimulated in vitro with autologous tumor cells in the presence of IL-2, they proliferate and differentiate into cytolytic T lymphocytes (CTL)[1] that specifically recognize the melanoma cells (1). A large panel of tumor-specific MHC class I–restricted CTL clones derived from the effector cells were shown to recognize at least six different antigens on melanoma line MZ2-MEL (2). This multiplicity of antigens recognized by autologous CTL clones was also observed with two other melanoma lines that could be studied in detail, SK29-MEL and LB33-MEL (3, 4).

Several antigens recognized by CTL on MZ2-MEL cells have been identified. Antigens MZ2-E and -Bb are two different peptides derived from a protein encoded by gene *MAGE-1* and presented by the MHC class I molecules HLA-A1 and HLA-Cw*1601, respectively (5–7). Antigen MZ2-D is a peptide also presented by HLA-A1, which is encoded by gene *MAGE-3* (8), another member of the *MAGE* gene family that comprises at least 12 highly homologous genes (9). Antigen MZ2-Ba is a peptide encoded by gene *BAGE* and presented by HLA-Cw*1601 (10). Antigen MZ2-F is a peptide encoded by gene *GAGE-1* and presented by HLA-Cw6 (11). Genes *MAGE*, *BAGE*, and *GAGE* are normal genes that are not expressed in normal tissues, with the exception of testis, but are expressed in a variety of tumors of different histological types. Antigen MZ2-C is a peptide encoded by the tyrosinase gene and presented by HLA-B44 (Brichard, V., J. Herman, A. Van Pel, C. Wildmann, B. Gaugler, T. Boon, and B. Lethé, manuscript in preparation). In contrast to the former group of genes, the tyrosinase gene is expressed in melanocytes as well as in melanoma cells.

Antigen MZ2-E or MAGE-1.A1, thus defined as the nonapeptide 161-169 derived from protein MAGE-1 and bound to HLA-A1 (6), was recognized by 7 out of a collection of 70 tumor-specific CTL clones derived from melanoma patient MZ2 (2). Moreover, when limiting numbers of MZ2 blood mononuclear cells (BMC) were stimulated with autologous tumor cells to estimate the frequency of precursors of antitumor CTL (CTL-P), 1/7,000 BMC were anti-MZ2-MEL-specific CTL-P (12), and

[1]*Abbreviations used in this paper:* BMC, blood mononuclear cell; CTL, cytolytic T lymphocytes; IASA, iodo, azido salicylic acid; $LY_{50}$, 50% of maximal lysis.


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~10% of them recognized peptide MAGE-1.A1 (Coulie, P. G., unpublished observations). Together, these data suggest that 10% of the antitumor CTL of patient MZ2 are specific for antigen MAGE-1.A1. We wished to analyze the diversity of this CTL response in terms of both the fine specificity of antigen recognition and of the primary structure of the TCR α and β chains.

## Materials and Methods

*Patient and Tumor Cell Line.* Melanoma cell line MZ2-MEL was established in 1982 from an adrenal metastasis of patient MZ2 (1). The patient remains disease free since 1983 after cytostatic chemotherapy and surgical removal of multiple lymph node and visceral metastases (Knuth, A., personal communication). She was repeatedly vaccinated with mutagenized and lethally irradiated autologous melanoma cell clones from 1983 on (2).

*Derivation and Culture of CTL Clones.* The obtention of anti-MZ2-E CTL clone 82/30 has been described previously (1). Clone 253/47 was obtained as follows. BMC of patient MZ2 were labeled with fluoresceinated anti-CD8β mAb 1A3.3 (a gift of Dr. D. Olive, Institut Paoli Calmette and Institut National de la Santé et de la Recherche Medicale Unité 211, Marseille, France), and $CD8^+$ lymphocytes were sorted on a flow cytometer (ATC3000; ODAM, Wissembourg, France). They were seeded (1,000/well) in V-bottom microwells (Nunc, Roskilde, Denmark) and stimulated with irradiated (100 Gy from a cesium source) autologous MZ2-MEL.3.1 melanoma cells (3,000/well) in a total volume of 100 μl of Iscove's medium (GIBCO BRL, Gaithersburg, MD) supplemented with L-arginine (0.55 mM), L-asparagine (0.24 mM), L-glutamine (1.5 mM), 2-ME ($5 \times 10^{-5}$ M), 10% human serum, 10 U/ml of human rIL-2 (a gift of Biogen, Geneva, Switzerland), and 5 U/ml of human rIL-4 (a gift from Dr. R. Devos, Roche Research Center, Ghent, Belgium). 1 U/ml of IL-2 is defined as the concentration that gives 50% maximal proliferation of CTLL-2. 1 U/ml of IL-4 is the concentration that gives 50% maximal proliferation of human T cells previously activated with PHA. On day 7, the microcultures were restimulated by addition of 100 μl of medium (of the same composition as on day 0) containing 3,000 irradiated tumor cells. On day 15, aliquots of the responder cells were transferred into other microwells to test their lytic activities. The remaining cells were restimulated as on day 7. On day 21, aliquots of responder cells were used again for lysis assays, and microcultures that exerted a strong anti-MZ2-E lytic activity were identified. The remaining responder cells from these selected clones, among them clone MZ2-CTL-253/47, were transferred on day 23 into 2-ml wells (Nunc) and were stimulated by the addition of irradiated autologous tumor cells ($10^5$/well) and irradiated LG2-EBV-B cells ($10^6$/well) as feeder cells, in medium identical to the previously described one except with a higher concentration of IL-2 (50 U/ml). Long-term culture of the CTL clone was pursued as described (1).

*Synthetic Peptides and CTL Assays.* Peptides were synthesized on solid phase using Fmoc for transient $NH_2$-terminal protection as described (8) and characterized by mass spectrometry. All peptides were >90% pure as indicated by analytical HPLC. Lyophilized peptides were dissolved in 20 mM PBS and stored at −20°C. Lysis of target cells by established CTL clones or by microcultures was tested by chromium release assay using standard procedures (8). Target cells were $^{51}Cr$ labeled for 1 h at 37°C in the presence of anti-human class I MHC mAb W6/32 (13) in the form of hybridoma culture supernatant and washed three times. Target cells (1,000 cells in 50 μl) were then incubated in V-bottom microwells in the presence of various concentrations of peptide (100 μl) for 15 min at room temperature. CTL were then added (10,000 cells in 50 μl), and chromium release was measured after 4 h at 37°C.

*Cloning and Sequencing of TCR.* Total RNA from $10^6$ cells of each clone was extracted as described (14) and reverse transcribed using a poly-dT primer and reverse transcription kit reagents (Boehringer Mannheim, Mannheim, Germany). Aliquots of the cDNA were amplified by PCR in separate tubes using a panel of Vα- or Vβ-specific oligonucleotides and a Cα- or Cβ-specific oligonucleotide (15). PCR amplification was carried out on an automate (9600; Perkin-Elmer Cetus Instruments, Norwalk, CT) and comprised a 30-s denaturation step at 94°C, followed by 40 cycles of 25 s at 94°C, 45 s at 60°C, and 45 s at 72°C. 10 μl of PCR products was loaded on a 2% agarose gel and electrophoresed. Amplified DNA was electroeluted and either directly sequenced (16) or cloned in the pCRTM vector according to the TA cloning kit procedures (Invitrogen, San Diego, CA). Upon electroporation in *Escherichia coli* OneShot INVαF′ strain (Invitrogen), plasmid DNA of recombinant colonies was prepared (QIAGEN Inc., Chatsworth, CA) and sequenced using the Sequenase kit (2.0; United States Biochemical, Cleveland, OH).

*Modeling of the HLA-A1/MAGE-1.A1 Peptide Complex.* The structure of the complex was modeled using the ProMod package developed by M. Peitsch (17). Briefly, an averaged framework was constructed from the experimentally defined structures of cocrystals of HLA-A2 with defined antigenic peptides (18), of HLA-B27 (19), of HLA-A68 with an influenza peptide (20), and of $H\text{-}2K^b$ (21) (Brookhaven Protein Databank, Brookhaven National Laboratory, Upton, NY). The aligned HLA-A1 sequence was fitted on this framework, and the most likely positions of polymorphic amino acid side chains were determined by scanning for sterically unhindered conformations consistent with local secondary structure. The initial conformation of the MAGE-1.A1 peptide backbone was similarly predicted from those of influenza matrix M1 58-66, HTLV-1 Tax 11-19, hepatitis B nucleocapsid 18-27, and HIV gp120 197-205 peptides bound to HLA-A2 (18). The optimal position of the peptide within the binding groove was calculated by rigid body minimization, and the HLA–peptide complex was then relaxed without constraints by 500 steps of Powell minimization. The stability of the final calculated conformation was verified by simulated annealing after heating to 300°K, using standard molecular dynamics methods. Energy minimizations were carried out using the X-PLOR program (Axel Brünger, Yale University, New Haven, CT).

## Results

When peripheral blood lymphocytes of melanoma patient MZ2 were stimulated in vitro with irradiated autologous tumor cells in the presence of IL-2, a strong lymphocyte proliferation was observed that was accompanied by the development of a cytolytic activity directed against the stimulator cells (1). The effector lymphocytes were cloned by limiting dilution, and several CTL clones were derived that showed specificity for the autologous melanoma clonal line MZ2-MEL.43. One of these CTL clones, MZ2-CTL-82/30, was shown to recognize one of the several antigens expressed by the tumor cells and named antigen MZ2-E (2). CTL 82/30 recognizes the nonapeptide MAGE-1.A1,



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encoded by gene *MAGE-1* and presented by HLA-A1 molecules (5, 6).

*Derivation of New Anti–MAGE-1.A1 CTL from Patient MZ2.* To study the diversity of anti–MAGE-1.A1 CTL in melanoma patient MZ2, we derived other anti–MAGE-1.A1 CTL clones from blood lymphocytes obtained from the patient 8 yr after the derivation of CTL 82/30. Several hundred microcultures were set up in which limited numbers of $CD8^+$ lymphocytes were stimulated either with irradiated autologous tumor cells (experiment 253) or with autologous BMC incubated with peptide MAGE-1.A1 (experiment 258), in the presence of IL-2. After three or four rounds of weekly stimulation, the microcultures were tested for their ability to lyse the MHC class I–negative B cell line C1R transfected with *HLA-A1* and incubated with peptide MAGE-1.A1. The lytic activities of CTL clones 253/47 and 258/8 are shown in Fig. 1, together with that of CTL clone 82/30. All three CTL lyse MZ2-MEL.43 cells but not the $E^-$ antigen–loss variant MZ2-MEL.2.2 cells, which do not express gene *MAGE-1*. However, the three CTL lyse $E^-$ cells incubated with peptide MAGE-1.A1, or $E^-$ cells transfected with gene *MAGE-1*. These results indicate that the three CTL recognize the same antigen.

*Absence of Recognition of Homologous MAGE Peptides by Anti–MAGE-1.A1 CTL.* Gene *MAGE-1* belongs to a family of at least 12 related genes, of which 6 are expressed in a number of tumors of various histological types (9). Several of the MAGE polypeptide sequences contain nonapeptides with various degrees of homology to the antigenic MAGE-1.A1 peptide (Table 1). With the exception of the MAGE-2 peptide, all these peptides contain the HLA-A1 binding motif consisting of Asp in position 3 and Tyr in position 9 (22–24). Using a competition assay, we found that, with the exception of the MAGE-2 peptide, the peptides listed in Table 1 could indeed efficiently bind to HLA-A1 (8), but none of them sensitized HLA-$A1^+$ target cells to lysis by any of the three anti–MAGE-1.A1 CTL clones, illustrating the exquisite specificity of these CTL for the MAGE-1.A1 peptide (not shown).

**Table 1.** *MAGE Nonapeptides Homologous to the MAGE-1 Antigenic Peptide*

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| MAGE-1 | E | A | D | P | T | G | H | S | Y |
| MAGE-2 | E | V | V | P | I | S | H | L | Y |
| MAGE-3 | E | V | D | P | I | G | H | L | Y |
| MAGE-4a | E | V | D | P | A | S | N | T | Y |
| MAGE-4b | E | V | D | P | T | S | N | T | Y |
| MAGE-6 | E | V | D | P | I | G | H | V | Y |

Amino acid sequences corresponding to MAGE-1 161-169, MAGE-2 168-176, MAGE-3 168-176, MAGE-4a 168-176, MAGE-4b 169-177, and MAGE-6 168-176 (9).

*Differential Recognition of MAGE-1.A1/MAGE-3.A1 Hybrid Peptides.* HLA-A1–restricted CTL clones were obtained that recognize a MAGE-3 peptide, designated MAGE-3.A1, that is homologous to the MAGE-1.A1 peptide (8). As mentioned above, the anti–MAGE-1.A1 CTL do not recognize peptide MAGE-3.A1, and the anti–MAGE-3.A1 CTL conversely failed to recognize peptide MAGE-1.A1 (8). Peptides MAGE-1.A1 and MAGE-3.A1 differ at positions 2, 5, and 8 (Table 1).

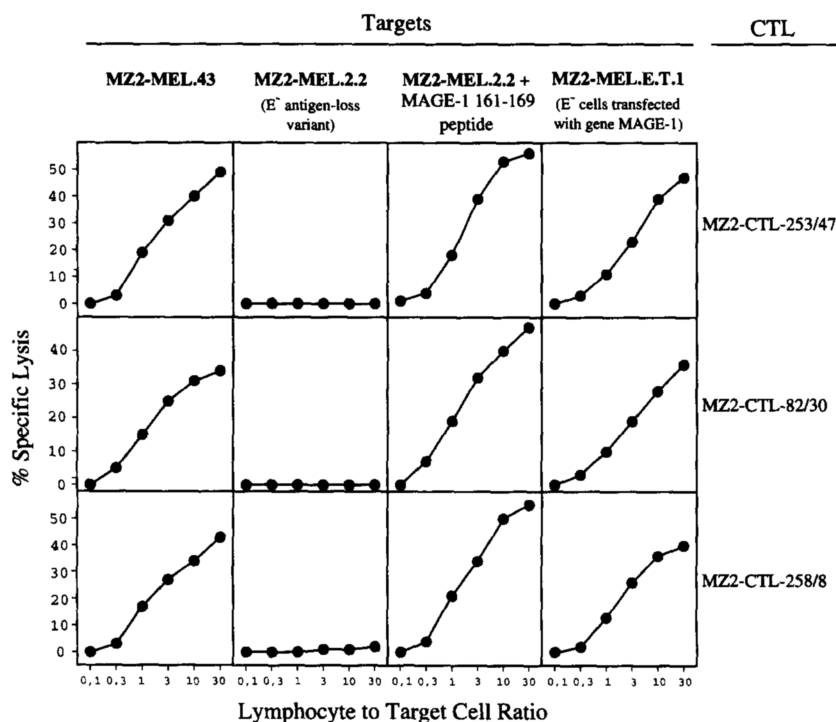


**Figure 1.** Lytic activities of three independent anti–MAGE-1.A1 CTL clones of melanoma patient MZ2. Anti–MAGE-1.A1 CTL clone MZ2-CTL-82/30 was obtained previously by stimulating blood lymphocytes of patient MZ2 with autologous melanoma cells. CTL clones 253/47 and 258/8 were obtained by stimulating $CD8^+$ blood lymphocytes with autologous tumor cells or with autologous BMC incubated with peptide MAGE-1.A1, respectively. Target cells included the clonal melanoma line MZ2-MEL.43, the $E^-$ antigen–loss variant cells MZ2-MEL.2.2, which lost expression of gene *MAGE-1*, the $E^-$ cells in the presence of 1 μM of peptide MAGE-1.A1, and the clonal cell line MZ2-MEL.ET.1, obtained after transfection of $E^-$ cells with gene *MAGE-1*.



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**Table 2.** *Recognition of Variant MAGE-1 Peptides by Different CTL Clones*

| CTL clone | MAGE-1 161-169 | Relative antigenic activity of variant MAGE-1 peptides* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | E161 | D163 | P164 | T165 | G166 | H167 | S168 | Y169 |
| | *[pM] 50%‡* | | | | | | | | |
| **253/47** | **100** | **<0.0001** | <0.0001 | 0.3 | 0.02 | <0.0001 | <0.0001 | 0.5 | <0.0001 |
| **82/30** | **100** | 0.4 | <0.0001 | **<0.0001** | 0.003 | 0.0001 | 0.01 | 5 | 0.0006 |
| **258/8** | **130** | 1 | <0.0001 | 1 | 0.02 | <0.0001 | <0.0001 | **<0.0001** | <0.0001 |

*The amino acid residue on top of each column (single-letter code) represents the residue substituted by Ala. The antigenic activity for both the eight single-Ala substituted MAGE-1 nonapeptides and the unsubstituted MAGE-1 peptide was determined in a $^{51}$Cr-release assay. The lymphocyte to target cell ratio was 10:1. Threefold dilutions for each peptide were made in a concentration range from $10^{-6}$ to $10^{-12}$ M. The relative antigenic activity is calculated as the concentration of the reference peptide MAGE-1.A1 161-169 required to obtain $LY_{50}$ divided by that of the variant peptide.

‡Numbers represent the concentration (picomolar, $10^{-12}$M) of the nonsubstituted MAGE-1.A1 161-169 nonapeptide required to obtain $LY_{50}$ by the corresponding CTL clones. This value was used as the reference concentration for calculation of the relative potency of all the other peptides included in this table.

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MAGE-1.A1/MAGE-3.A1 hybrid peptides were synthesized that incorporated one or two substitutions at P2, P5, or P8. These hybrid peptides displayed similar HLA-A1–binding activities. They were tested for recognition by the anti–MAGE-1.A1 CTL on cells of the MHC class I–negative lymphoblastoid cell line C1R transfected with *HLA-A1* (Fig. 2). For CTL clone 253/47, all substitutions abrogated recognition, with the exception of substitution of Leu for Ser at P8. In contrast, for CTL clone 258/8, the only tolerated substitution was the replacement of Val for Ala at P2. For CTL clone 82/30, the pattern of recognition was more complex. Similar to CTL 253/47, CTL 82/30 recognized the substitution at P8, but it also recognized the substitution at P2, as well as, albeit with a lower efficiency, the simultaneous substitutions at P2 and P8.

These results indicate that the three anti–MAGE-1.A1 CTL displayed different fine specificities. In spite of these differences, all three clones failed to recognize any of the three hybrid peptides containing the substitution at P5. Thus, the residue at P5 is critical for discrimination of the MAGE-1.A1 from the MAGE-3.A1 epitope.

*Differential Recognition of MAGE-1.A1 Variant Peptides Incorporating Ala Substitutions.* The anti–MAGE-1.A1 CTL were tested on a panel of MAGE-1.A1 variant peptides that incorporated single Ala substitutions at each position. The effect of Ala substitution can be equated to the removal of most of the side chain at a given position without disturbing the overall peptide configuration. The substituted peptides bound to HLA-A1 (8), with the exception of those with Ala at positions 3 or 9, which were shown to anchor antigenic peptides in HLA-A1 molecules (22–24).

The relative antigenic activities of the substituted peptides were measured for each CTL (Table 2). Serial dilutions of each peptide were used to sensitize C1R-A1 cells to lysis by CTL, and the concentration of peptide required to obtain 50% of maximal lysis ($LY_{50}$) was determined. To compare the $LY_{50}$ of a given peptide for different CTL clones, we expressed the results as relative antigenic activities, which are the ratios between the $LY_{50}$ of the original peptide and that of the variant peptide. As expected, substitutions at HLA-A1 anchor positions 3 and 9 abrogated recognition by all three CTL. Substitutions at P5, P6, and P7 greatly reduced the efficiency of recognition by all three CTL as well. But other substitutions, substitution at P1 for CTL 253/47, at P4 for CTL 82/30, and at P8 for CTL 258/8, proved to abrogate the recognition by only one of the CTL (Table 2 and Fig. 3). Remarkably, the recognition of these substitutions was mutually exclusive. These results

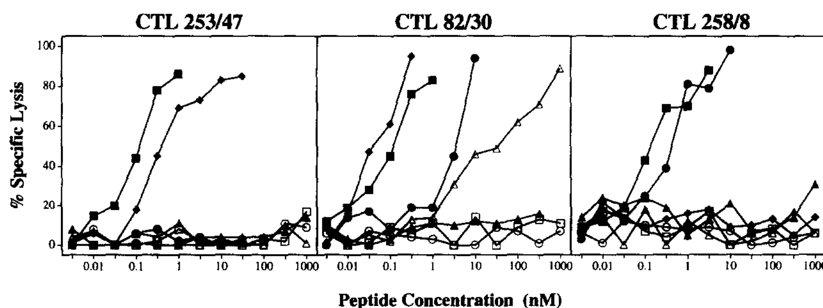


**Figure 2.** Recognition of MAGE-1.A1/MAGE-3.A1 hybrid peptides by the anti–MAGE-1.A1 CTL clones. $^{51}$Cr-labeled C1R-A1 target cells were incubated with the indicated concentrations of the hybrid MAGE peptides and with the CTL clones at a lymphocyte to target cell ratio of 10:1 for 4 h at 37°C. The hybrid peptides are designated by a code that indicates which substitution was introduced in the sequence; the number is the position of the substitution, the first letter designates the residue at that position in the original MAGE-1.A1 peptide, and the second letter designates the new residue at that position in the substituted peptide. -■-, EADPTGHSY; -●-, A2V; -▲-, T5I; -◆-, S8L; -□-, A2V + T5I; -○-, T5I + S8L; -△-, A2V + S8L.

**Table 3.** *TCR Usage among Human CTL Specific for Tumor Antigen MZ-2E*

| | TCR α chain CDR3-like coding region | TCR β chain CDR3-like coding region |
|---|---|---|
| CTL 253/47 | Vα1-Jα39* | Vβ7-Dβ1-Jβ1.1 |
| | 93‡ … 104 | 95 … 106 |
| | TGT GTT GTG AGT G AT AAT GCA GGC AAC ATG CTC ACC TTT | TGC GCC AGC CAA GAT CTT TTC AG G AAC ACT GAA GCT TTC TTT |
| | C V V S D N A G N M L T F | C A S Q D L F R N T E A F F |
| CTL 82/30 | Vα12-Jα39 | Vβ1-Dβ2-Jβ2.3 |
| | 93‡ … 104 | 95 … 106 |
| | TGT GCT CTG GGA GGG G TG AAT AAT AAT GCA GGC AAC ATG CTC ACC TTT | TGT GCC AG C AAC ATA GCG GGC GGG AGT T AT ACG CAG TAT TTT |
| | C A L G G V N N N A G N M L T F | C A S N I A G G S Y T Q Y F |
| CTL 258/8 | Vα2-Jα43 | Vβ4-Dβ2-Jβ2.1 |
| | 93‡ … 104 | 95 … 106 |
| | TGT GCC GTG GCA AAC AAT GAC ATG CGC TTT | TGC AGC GTT TTA GCG GGA GGG GGC T AT GAG CAG TTC TTC |
| | C A V A N N D M R F | C S V L A G G G Y E Q F F |

*Jα gene segment nomenclature was derived from Genbank entry HUMTCRADCV by Koop, B. F. et al. Jα39 is also known as JαAC17.
‡CDR3-like boundaries are indicated above the nucleotide sequence. Definition of these are according to Chothia, C. et al. (25) and encompass residues 93 to 104 for the α chain and residues 95 to 106 for the β chain.



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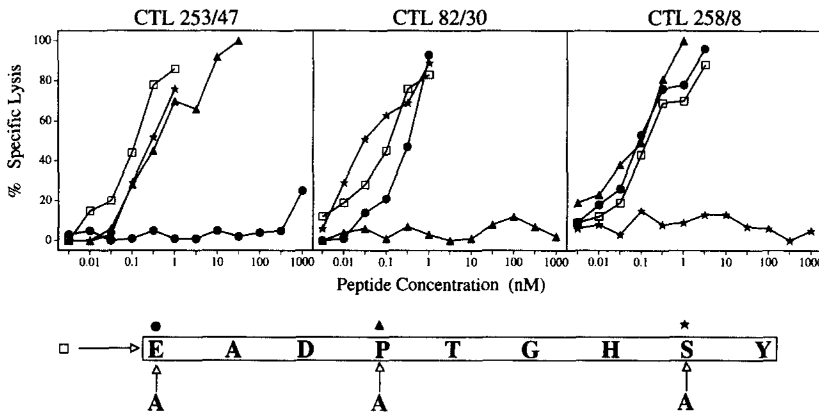


**Figure 3.** Recognition of MAGE-1.A1 variant peptides with single Ala substitutions by the anti–MAGE-1.A1 CTL clones. $^{51}$Cr-labeled C1R-A1 target cells were incubated with the indicated concentrations of peptide MAGE-1.A1 (□) or with analogues incorporating one Ala substitution at a time (●, ▲, or ★), and with the CTL clones at a lymphocyte/target cell ratio of 10:1 for 4 h at 37°C.

indicate that at least two regions of peptide MAGE-1.A1 are critical for recognition by the three CTL. The first, recognized by all CTL, spans residues at positions 5, 6, and 7. The other, different for each CTL, is constituted by residue P1, P4, or P8.

*Sequences of the TCR Used by Anti–MAGE-1.A1 CTL Clones.* As expected from the specificity analysis of the three CTL, each of them expressed a different TCR (Table 3). The gene segments used for both the α and the β chains of the TCR proved to differ totally from one CTL to another, with the exception of a Jα39 segment used by both CTL 253/47 and 82/30. Moreover, the primary structures of the three TCR do not share any common feature, neither in the sizes of their CDR3 equivalent regions nor in their amino acid composition.

*Distribution of the Different Anti–MAGE-1.A1 Specificities in Short-term Cultures of MZ2 Blood CTL.* To derive a new panel of independent anti–MAGE-1.A1 CTL from patient MZ2, 600 miniature mixed lymphocyte tumor cell cultures were set up with $CD8^+$ blood lymphocytes stimulated with autologous melanoma cells in the presence of IL-2 and IL-4. These lymphocytes were collected from the

**Table 4.** *Distribution of the Fine Specificities of Anti-MAGE-1.A1 CTL in Short-Term Cultured MZ2 Blood Lymphocytes*

| A. Targets | Lysis by microcultures (%) | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C1R-A1+MAGE-1.A1 | 5 | 7 | 2 | 5 | 0 | 0 | 0 | 1 | 70 | 5 | 0 | 0 | 8 | 2 | 3 | 4 | 9 | 0 | 3 | 1 | 57 | 3 | 1 | 0 |
| C1R-A1+variant E1A | 5 | 0 | 1 | 3 | 0 | 5 | 1 | 0 | 69 | 2 | 0 | 0 | 4 | 0 | 5 | 1 | 0 | 3 | 0 | 0 | 8 | 1 | 4 | 4 |
| C1R-A1+variant P4A | 3 | 5 | 3 | 3 | 3 | 0 | 0 | 0 | 68 | 0 | 0 | 0 | 9 | 0 | 0 | 1 | 0 | 2 | 2 | 0 | 58 | 0 | 0 | 0 |
| C1R-A1+variant S8A | 2 | 0 | 1 | 3 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 4 | 4 | 0 | 1 | 3 | 0 | 7 | 0 | 0 | 0 |
| K562 | 5 | 2 | 1 | 2 | 1 | 0 | 1 | 0 | 0 | 2 | 0 | 0 | 5 | 1 | 1 | 2 | 5 | 3 | 4 | 0 | 2 | 0 | 0 | 1 |

| B. | Peptides | | | | |
|---|---|---|---|---|---|
| | | MAGE-1.A1 peptide variant | | | |
| Number of microcultures | MAGE-1.A1 | E161A | P164A | S168A | Specificity |
| 2 | + | − | + | + | = CTL 253/47 |
| 7 | + | + | − | + | = CTL 82/30 |
| 2 | + | + | + | − | = CTL 258/8 |
| 4 | + | − | + | − | = other |
| 5 | + | + | + | + | = other |

(*A*) 600 microcultures were set up with sorted $CD8^+$ blood lymphocytes from patient MZ2 (1,000 cells/well) stimulated with irradiated autologous melanoma cells MZ2-MEL.43 (3,000 cells/well) in the presence of IL-2 (10 U/ml) and IL-4 (5 U/ml). Microcultures were restimulated on day 7 and 14 with stimulator cells, IL-2, and IL-4. On day 21, the lytic activities of aliquots of all microcultures were tested on the indicated targets, in the presence of a 50-fold excess of unlabeled K562 cells to inhibit the NK-like lytic activity. The lytic activities of 24 microcultures are shown. (*B*) The 20 microcultures with a lytic activity against peptide MAGE-1.A1 (>15% lysis) were classified according to their pattern of lysis of C1R-A1 cells incubated with the variant peptides. The lytic activity against a variant peptide is reported as + when it exceeded 50% of that measured against peptide MAGE-1.A1.



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patient 1 yr after those from which CTL 253/47 and 258/8 were derived, and 7 yr after those from which CTL 82/30 was derived. After three rounds of weekly restimulation, all microcultures were tested for their lytic activity against C1R-A1 cells previously incubated with peptide MAGE-1.A1 or with the three variant peptides incorporating Ala substitutions at positions P1, P4, or P8. An example of the lysis exerted by a small number of these microcultures is shown in Table 4 *A*. Among all microcultures, 20 that contained an anti–MAGE-1.A1 CTL could easily be identified. These CTL could be classified on the basis of their pattern of recognition of the three variant MAGE-1.A1 peptides (Table 4 *B*). Two, seven, and two CTL displayed a fine specificity corresponding to that of CTL 253/47, 82/30, and 258/8, respectively. This indicated that these three types of CTL were consistently present in different batches of MZ2 blood lymphocytes, with a higher proportion of the 82/30 type. Two additional fine specificities were identified among the nine other CTL, indicating that the repertoire of anti–MAGE-1.A1 CTL in patient MZ2 may actually comprise at least five independent fine specificities.

## Discussion

Our results indicate that the repertoire of CTL directed against tumor antigen MAGE-1.A1 in melanoma patient MZ2 is diverse both in terms of recognition of antigen and of TCR usage. The diversity of recognition of antigen was demonstrated for three independent CTL clones by analyzing their recognition of hybrid MAGE-1.A1/MAGE-3.A1 peptides and of a set of MAGE-1.A1 peptide analogues containing single alanine substitutions. The results indicated that three residues (P5, P6, and P7) of peptide MAGE-1.A1 were essential for recognition by all three CTL, whereas each of residues at P1, P4, or P8 were relevant for only one CTL. Clones expressing these three distinct fine specificities of recognition could be derived from blood samples obtained from patient MZ2 several years apart, suggesting that they were stable members of the patient's T cell repertoire. Moreover, testing additional anti–MAGE-1.A1 CTL clones for recognition of some of the Ala-substituted peptides indicated that the repertoire may actually comprise additional anti–MAGE-1.A1 specificities, which we did not characterize further.

Attempting to visualize how CTL 253/47, 82/30, and 258/8 TCR could interact with their antigen, we generated a molecular model of peptide MAGE-1.A1 bound to the HLA-A1 molecule. Model building was based on the structures of cocrystals of HLA class I molecules with defined antigenic peptides. At the carboxy terminus of the peptide, the side chain of Tyr-P9 is buried in the F pocket of the HLA-A1 peptide-binding site, and the hydroxyl group of its aromatic ring presumably makes a hydrogen bond with the carboxyl group of Asp116 from the floor of the groove. The side chain of Asp-P3 also points down into the groove, and the model predicts that its carboxylate group is available for a salt bridge with the positively charged side chain of HLA-A1 Arg114 present in the β2 strand of the floor of the groove. Peptide residues Glu-P1, Pro-P4, Thr-P5, Gly-P6, His-P7, and Ser-P8 side chains appear to be more accessible for direct interaction with the TCR (model not shown).

On the basis of this model, it is conceivable that the TCR of the three CTL clones analyzed in this study interact with the antigenic complex in distinct manners. Thus, the CTL 253/47 TCR might focus on the peptide portion comprised between its amino terminus and residue His-P7, and ignore changes affecting the side chain of the residue at P8. Conversely, the CTL 258/8 TCR might focus on the carboxy terminus of the peptide and ignore changes affecting the amino-terminal residues at P1 or P2. Finally, the CTL 82/30 TCR might focus on the middle portion of the peptide, and thus might not be sensitive to changes affecting the residues at both P1 or P2 and P8. In support of this interpretation, we recently observed that a MAGE-1.A1 variant peptide incorporating the photoactivatable derivative iodo, azido salicylic acid (IASA) at P1 was efficiently recognized by CTL 258/8 but not by CTL 253/47. In contrast, a peptide with IASA at P8 could be recognized by CTL 253/47 but not by CTL 258/8. Moreover, both variant peptides were recognized by CTL 82/30 (Luescher, I. F., and P. Romero, unpublished observations). Since the IASA group is approximately twice as bulky as the side chain of a tyrosine residue, these observations highlight the apparent lack of influence of the nature of the side chains at positions 1 or 8 on the efficiency of peptide recognition by different CTL clones. They suggest that the different TCR studied here may indeed have different "footprints" on the antigenic ligand.

The diversity of recognition by CTL is associated with a diverse TCR gene usage. However, somewhat surprisingly, the structural diversity of TCR appears here to be very high, inasmuch as the CDR3 sequences differ widely from one TCR to another. In another study in which CTL clones specific for an influenza peptide associated with HLA-B27 were also differentially sensitive to conservative and nonconservative amino acid substitutions at peptide positions 1, 4, and 8, their TCR were found to be less diverse (26, 27).

Detailed analyses of murine TCR repertoires specific for a variety of antigenic peptides presented by class I or class II MHC molecules have shown that they can be either relatively homogeneous, in terms of the germline V, (D), and J gene segments used and of the primary structure of the junctional regions (28–34), or, on the contrary, highly diverse (35–38). The differences did not depend on the nature of the presenting MHC molecule alone, since both homogeneous and diverse TCR repertoires were found in collections of CTL clones recognizing two unrelated antigenic peptides presented by the same MHC class I molecule (34, 36). In at least two of these studies, the diversity of TCR usage was correlated with a multiplicity of epitopes recognized on the same MHC–peptide complex (35, 39). Human CTL repertoires specific for class I–restricted viral peptides were recently shown to be very limited; similar or even identical α/β TCR primary structures were found in



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different individuals sharing the presenting HLA allele (26, 40–43).

The reasons for these so far unpredictable degrees of diversity of TCR repertoires are not yet well understood, and at least two types of explanations have been proposed. On one hand, it was suggested that there could be a relationship between the limited diversity of the repertoire against a given peptide and homologies of its sequence with a self-peptide. In this context, tolerance would greatly limit the diversity of the repertoire specific for antigenic peptides homologous to self (44). On the other hand, limited TCR diversity could be the result of a continuous selection of certain TCR during persistent infections such as those caused by HIV (41) or EBV (42). Along these lines, it has also been proposed that the limited heterogeneity of the TCR expressed by "public" clones, which are reproducibly isolated from different individuals in certain experimental models, would be the hallmark of an antigen-selected high affinity T cell response. Accordingly, such public clones would not be encountered in the repertoire specific for a self-antigen where, due to tolerance mechanisms, a low affinity T cell response would be expected (45).

Peptide MAGE-1.A1 is a self-peptide, but gene *MAGE-1* appears not to be expressed in adult normal tissues, with the exception of testis. It is a likely possibility that it is expressed in testis by germinal cells that do not express class I molecules. Whether gene *MAGE-1* is expressed earlier in development in such a way that peptides derived from MAGE proteins could contribute to shaping the immunological repertoire is unknown.

The degree of diversity of TCR repertoires specific for tumor antigens (46, 47) is relevant to the development of specific cancer immunotherapy that consists of immunizing cancer patients against defined antigens expressed on their tumor cells. One may envision that tumor-specific CTL expressing a high-affinity TCR might out-compete other clones, thus leading to the establishment of a highly restricted but potent antitumor T cell response that could be monitored in vitro by TCR usage analysis. Alternatively, the broad repertoire of anti–MAGE-1.A1 CTL specificities may prove to be a general phenomenon. If this is the case, it decreases the possibility for a tumor to escape through small antigenic variations and augments the possibility of immunized patients to mount a successful tumor rejection response.

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Address correspondence to Dr. Pedro Romero, Ludwig Institute for Cancer Research, Ch. des Boveresses 155, CH-1066 Epalinges, Switzerland.

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