[CANCER RESEARCH 61, 5850–5856, August 1, 2001]

Heterogeneous T-Cell Response to MAGE-A10$_{254–262}$: High Avidity-specific Cytolytic T Lymphocytes Show Superior Antitumor Activity[1]

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ABSTRACT

MAGE-encoded antigens, which are expressed by tumors of many histological types but not in normal tissues, are suitable candidates for vaccine-based immunotherapy of cancers. Thus far, however, T-cell responses to MAGE antigens have been detected only occasionally in cancer patients. In contrast, by using HLA/peptide fluorescent tetramers, we have observed recently that CD8$^+$ T cells specific for peptide MAGE-A10$_{254–262}$ can be detected frequently in peptide-stimulated peripheral blood mononuclear cells from HLA-A2-expressing melanoma patients and healthy donors. On the basis of these results, antitumoral vaccination trials using peptide MAGE-A10$_{254–262}$ have been implemented recently. In the present study, we have characterized MAGE-A10$_{254–262}$-specific CD8$^+$ T cells in polyclonal cultures and at the clonal level. The results indicate that the repertoire of MAGE-A10$_{254–262}$-specific CD8$^+$ T cells is diverse both in terms of clonal composition, efficiency of peptide recognition, and tumor-specific lytic activity. Importantly, only CD8$^+$ T cells able to recognize the antigenic peptide with high efficiency are able to lyse MAGE-A10-expressing tumor cells. Under defined experimental conditions, the tetramer staining intensity exhibited by MAGE-A10$_{254–262}$-specific CD8$^+$ T cells correlates with efficiency of peptide recognition so that "high" and "low" avidity cells can be separated by FACS. Altogether, the data reported here provide evidence for functional diversity of MAGE-A10$_{254–262}$-specific T cells and will be instrumental for the monitoring of peptide MAGE-A10$_{254–262}$-based clinical trials.

INTRODUCTION

A large body of experimental evidence accumulated in recent years strongly indicates that tumor cells specifically express Ags[3] that, under still poorly defined circumstances, can make them spontaneously immunogenic *in vivo*. Whereas both natural humoral (1) and cellular (2) specific immune responses against tumors can be detected in cancer patients, it is generally admitted that tumor Ag-specific CD8$^+$ CTLs constitute the primary effector arm of the adaptive immune responses against tumors. Therefore, recent efforts for the development of effective cancer vaccines are aimed at the elicitation or at the enhancement of specific CD8$^+$ T-cell responses to tumor Ag. Several groups of self-derived tumor Ags have been identified (2): (*a*) Ag encoded by mutated genes, *e.g.*, *p53* (3), *CDK4* (4); (*b*) Ag derived from tumor associated viruses, *e.g.*, HPV and EBV (5, 6); (*c*) overexpressed Ag, *e.g.*, HER2/neu (7); (*d*) tissue-specific differentiation Ag-specific to the tissue type from which the tumor, *e.g.*, Melan-A, gp100, and tyrosinase (8–10) expressed in melanomas and in cells of the melanocytic lineage but not in other normal cells; (*e*) nonmutated self Ag products with highly restricted tissue distribution expressed by a significant proportion of tumors of different histological types but not expressed in most somatic tissues, usually with the exception of the testis and therefore called cancer testis antigens. Examples of Ags in this group are SSX2 (11), NY-ESO-1 (12), and the Ags encoded by the *MAGE* genes (2).

The potency of antitumor CTL responses directed against peptide antigenic sequences derived from tumor Ags is influenced by parameters such as frequency and specificity of Ag expression in tumors, frequency of specific T cells, and avidity of specific CTLs for the tumor target (13, 14). Whereas CTL responses to most of the Ag groups listed above are relevant to a specific type of tumor, cancer testis Ags are ideal candidates for generic vaccination of cancers patients. We and others (15, 16) have reported recently that natural CTL responses to the cancer testis Ag NY-ESO-1 can be detected in melanoma patients as well as in patients bearing other types of tumors. In the case of MAGE-encoded Ags, however, despite the identification of numerous CTL-defined antigenic peptides derived from individual *MAGE* gene products (2), specific CTLs have been thus far detected only rarely in cancer patients. Thus, both the extent and the nature of MAGE Ag-specific CTL repertoire have remained unexplored.

MAGE-A10 is a nuclear protein (17) expressed by tumors of different histological types, with the highest frequencies (33–50%) observed in melanoma, bladder carcinoma, lung carcinoma, esophageal, and head and neck squamous carcinoma. Recently, an HLA-A2-restricted MAGE-A10 encoded epitope recognized by a CTL clone from a melanoma patient has been found to correspond to peptide 254–262 (18). We have observed recently that, in contrast to previously analyzed CD8$^+$ T cells, responses to other MAGE antigenic peptides, CD8$^+$ T cells specific for peptide MAGE-A10$_{254–262}$ are found frequently in peptide-stimulated PBMCs from A2-expressing melanoma patients and healthy donors (19). Therefore, peptide MAGE-A10$_{254–262}$ is currently an important candidate vaccine for immunotherapy of cancer. In this perspective, however, a deeper knowledge of the functional diversity of MAGE-A10$_{254–262}$-specific T cells as well as the correlation of the latter with tumor recognition and, possibly, with TCR structural diversity, would be instrumental for evaluating the efficacy of vaccination trials aimed at eliciting MAGE-A10$_{254–262}$-specific, tumor-reactive T cells. With this aim, in the present study we have characterized MAGE-A10$_{254–262}$-specific T cells derived from six melanoma patients and two healthy donors in terms of avidity of Ag recognition and specific tumor lysis. On the basis of the analysis of specific clonal populations, we observed that MAGE-A10$_{254–262}$-specific T cells can recognize peptide MAGE-A10$_{254–262}$ within a relatively large range of avidity. Importantly, MAGE-A10-specific T cells able to recognize peptide MAGE-A10$_{254–262}$ with high avidity (comparable with that of T cells specific for virally derived Ags) showed a superior capacity to lyse MAGE-

Received 2/13/01; accepted 5/31/01.

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[1] Supported in part by Grant SKL 782-2-1999 from the Swiss Cancer League (to V. R-G.).

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[3] The abbreviations used are: Ag, antigen; PBMC, peripheral blood mononuclear cell; TCR, T-cell receptor; mAb, monoclonal antibody; FACS, fluorescence-activated cell sorting.

A10-expressing tumors. In addition, we observed that, under defined experimental conditions, the brightness of specific T-cell staining with A2/MAGE-A10$_{254-262}$ peptide tetramers (tetramers hereafter) correlate with avidity of Ag recognition thus that high and low avidity MAGE-A10$_{254-262}$-specific T cells can be separated by FACS.

MATERIALS AND METHODS

Cells. The melanoma cell line Me 275 was generated in our laboratory from a surgically excised melanoma metastasis from patient LAU 50. The melanoma cell line NA-8 was kindly provided by Dr. F. Jotereau (U211, Institut National de la Santé et de la Recherche Médicale, Nantes, France). Polyclonal monospecific and monoclonal T-cell populations from stage III-IV melanoma patients and healthy donors were obtained as described elsewhere (16). Briefly, highly enriched CD8$^+$ T lymphocytes were stimulated with peptide MAGE-A10$_{254-262}$ (1 μM) and irradiated autologous CD8$^-$ cells as Ag-presenting cells in medium containing human recombinant interleukin 2 and human recombinant interleukin 7. One week later, cells were restimulated with peptide pulsed and irradiated T2 cells for an additional 6 days prior to tetramer analysis. Cultures were stained with A2/MAGE-A10 tetramers and anti-CD8 mAb at day 7 after the second peptide stimulation. CD8$^+$ tetramer$^+$ T cells were isolated by FACS and stimulated, either as polyclonal cultures or under limiting dilution conditions, in the presence of irradiated allogenic feeder cells, phytohemagglutinin, and human recombinant interleukin 2 (150 units/ml; Glaxo, Geneva, Switzerland, kindly provided by Dr. M. Nabholz, ISREC, Epalinges, Switzerland). T cells were cultured in Iscove's Dulbecco medium (Life Technologies, Inc., Basel, Switzerland) supplemented with 0.24 nM Asn, 0.55 nM Arg, 1.5 mM Gln, 8% pooled human A$^+$ serum (CTL medium).

Ag Recognition Assay. Ag recognition was assessed by chromium-release assay. Briefly, chromium-labeled target T2 cells (1000) were incubated in the presence of serial dilutions of parental peptide or analogues and effector cells at an effector:target cell ratio of 10:1. Chromium release was measured in the supernatant after 4 h of incubation at 37°C. The % specific lysis was calculated as:

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

The relative antigenic activity of alanine-substituted MAGE-A10 peptide analogues was calculated as the [nM] required to obtain 50% of maximal lysis with the parental MAGE-A10$_{254-262}$ GLYDGMEHL peptide divided by that of the analogue peptide. Specific tumor recognition was similarly assessed by using as target cells chromium-labeled Me 275 cells (HLA-A2$^+$ MAGE-A10$^+$).

Tetramers, mAbs, and BV Repertoire Analysis. HLA-A2/MAGE-A10$_{254-262}$ peptide tetramers were synthesized as described (20, 21) using peptide MAGE-A10$_{254-262}$. Anti-CD8^{FITC} and anti CD8^{PerCP} were purchased from Becton Dickinson (San Jose, CA). To enumerate MAGE-A10-specific T cells in the cultures, samples were stained with tetramers^{PE} (0.1 μg/sample in 20 μl) for 1 h at room temperature and then with anti-CD8^{FITC} for an additional 30 min at 4°C. For BV repertoire analysis, a panel of 21 anti-BV mAbs (purchased from Immunotech, Beckman-Coulter, Marseilles, France) was used. Anti-BV 5.3, -9, -18 and -23 were used as purified mAbs, whereas the remaining BV mAbs were FITC conjugated. Staining and washing were performed in PBS, 0.2% BSA, and 0.02% sodium azide. Cells were: (*a*) stained with tetramers-^{PE} (5 μg/ml) for 1 h at room temperature and washed; (*b*) with purified anti-BV mAbs for 20 min at 4°C and washed; (*c*) with goat antimouse FITC-labeled mAb for 30 min at 4°C and washed; (*d*) with IgG1 and IgG2a mAbs for 10 min at 4°C; and (*e*) with anti-CD8-^{PerCP} for 30 min at 4°C. Staining with directly labeled anti BV mAbs was similarly performed for steps 1, 2, and 5. After completing the staining, cells were washed once with the same buffer and analyzed in a FACSCalibur, FACScan, or FACSVantage (Becton Dickinson). Data analysis was performed using the Cell Quest software. For tetramer-guided cell sorting, the same protocol was used except for the fact that the buffer contained no sodium azide. For PCR analysis, total RNA was prepared from MAGE-A10$_{256-262}$-specific clones using TRIzol (Life Technologies, Inc., Paisley, United Kingdom) and converted to cDNA by standard methods using reverse transcriptase and an oligo(dT) primer. cDNA was then amplified using a panel of validated 5′ sense primers specific for the 24 BV subfamilies and 1 3′ antisense primer specific for the *BC* gene segment (22). TCR BV-BC PCR products were cloned into pBS-SK$^+$ vector (Stratagene, La Jolla, CA). Competent XL-1 blue *Escherichia coli* (Stratagene) were transformed and plated for blue/white color selection on medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Plasmid DNA was extracted from white colonies using the Qiagen Plasmid Mini kit (Qiagen, Hilden, Germany) and sequenced using the Dye Terminator cycle Sequencing kit (ABI PRISM; Perkin-Elmer, Foster City, CA) according to the manufacturer's instructions.

RESULTS

Generation of MAGE-A10-specific Polyclonal and Monoclonal T-Cell Populations from Melanoma Patients and Healthy Donors. We have shown previously that A2/MAGE-A10$_{256-262}$ tetramer$^+$ (tetramers hereafter) CD8$^+$ T cells are detected frequently among peptide-stimulated PBMCs from A2$^+$ melanoma patients and healthy donors (19). To gain more insight into MAGE-A10 antigen recognition by specific T lymphocytes, A2/MAGE-A10$_{256-262}$ tetramer$^+$ CD8$^+$ T cells from peptide-stimulated PBMCs of six melanoma patients and two healthy donors were isolated by tetramer guided cell sorting as described previously (23). The isolated cells were then expanded either as polyclonal lines (Fig. 1*A*) or under limiting dilution conditions to isolate T-cell clones. A total number of six polyclonal lines (from patients LAU 50, LAU 155, LAU 119, LAU 42, and healthy donors HD 591 and HD 795) were generated. Specific T-cell clones were obtained from LAU 50, LAU 155, LAU 203, LAU 169, LAU 42, and from HD 795.

Tetramer-guided Analysis of the TCR BV Chain Usage by MAGE-A10-specific Cells. TCR BV chain usage of MAGE-A10-specific cells in peptide-stimulated populations was assessed by triple staining with anti-CD8 mAb, tetramers, and a panel of 21 mAbs directed against the variable region of the TCR β chain as described previously (24). An example of the results obtained is shown in Fig. 1*B*. Anti-BV mAbs were also used to assess the BV segment usage of isolated clonal populations. Tables 1 and 2 shows the results of this

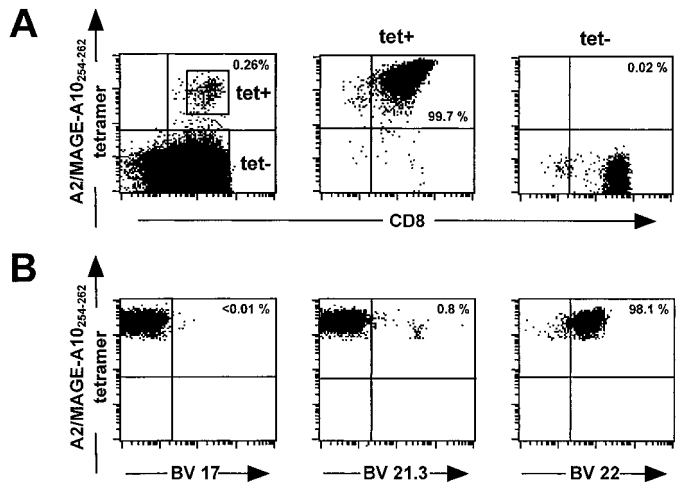

Fig. 1. Isolation of A2/MAGE-A10 tetramer$^+$ CD8$^+$ T cells from peptide-stimulated PBMCs and anti-BV mAb guided analysis of their BV gene segment usage. *A*, peptide-stimulated PBMCs were stained with A2/MAGE-A10 tetramers and anti-CD8 mAb as detailed in "Materials and Methods." Tetramer$^+$ CD8$^+$ (*tet+*) as well as tetramer$^-$ CD8$^+$ (*tet−*) T cells were isolated from peptide-stimulated PBMCs by FACS and further expanded *in vitro* by stimulation with phytohemagglutinin. *B*, after *in vitro* expansion, these polyclonal cultures were stained with A2/MAGE-A10 tetramers^{PE}, together with anti-BV^{FITC} and anti-CD8^{PerCP} mAbs. Numbers in the *upper right quadrant* represent the percentage of BV$^+$ tetramer$^+$ cells within the CD8$^+$ subset. Data in this figure were for peptide-stimulated PBMCs from melanoma patient LAU 50. The analysis was similarly performed in the case of five other A2$^+$, MAGE-A10$_{254-262}$ responder melanoma patients and two healthy donors.

Table 1 *Tetramer-guided analysis of the TCR BV chain usage of MAGE-A10$_{254-262}$-specific cells*

	LAU 50 P[a]	LAU 50/22	LAU 50/15	LAU 155 P[a]	LAU 155/6D1	LAU 119 P[a]	LAU 203/1B8	LAU 169/4E8	LAU 42 P[a]	LAU 42/2D6	HD 591 P[a]	HD 795 P[a]	HD 795/4D11	HD 795/2B6
BV-specific mAb														
1	<0.01[b]	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	63	100	<0.01	<0.01	<0.01	<0.01
13.1	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	30	<0.01	<0.01	<0.01	<0.01	<0.01
17	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	43	100	<0.01
21.3	0.8	<0.01	100	100	100	<0.01	<0.01	<0.01	<0.01	<0.01	21.7	<0.01	<0.01	<0.01
22	98	100	<0.01	<0.01	<0.01	100	<0.01	100	<0.01	<0.01	32	9.1	<0.01	<0.01
% repertoire covered	98.8			100		100			93		53.7	52.1		

[a] P, polyclonal. The others are CTL clones obtained by limiting dilution culture.
[b] Number represents percentage of positive cells within the A2/MAGE-A10$_{254-262}$ tetramer+ CD8+ T-cell fraction.

analysis for polyclonal cultures and representative clones. For 4 of the polyclonal cultures analyzed (LAU 50, LAU 155, LAU 119, and LAU 42), BV segment usage of >90% of tetramer$^+$ cells was determined by using this method. However, for cultures from healthy donors HD 591 and HD 795, BV usage of only ~50% of tetramer$^+$ cells was determined. Most of tetramer$^+$ cells in the polyclonal culture from LAU 50 expressed BV 22, whereas a small but significant proportion expressed BV 21.3 (Fig. 1*B*). Both BV 21.3 (clone LAU 50/15)- and BV 22-expressing clones (clone LAU 50/22) were isolated from this culture. Tetramer$^+$ cells in the polyclonal culture from patient LAU 155 expressed exclusively BV 21.3. Accordingly, a BV 21.3-expressing clone (LAU 155/6D1) was isolated from this culture. Tetramer$^+$ cells in the polyclonal culture from patient LAU 119 exclusively expressed BV 22. BV gene segment usage of a T-cell clone derived from patient LAU 203 (clone LAU 203/1B8) could not be determined by staining with anti BV antibodies and was determined by PCR analysis (BV 13; Table 2). A BV 22-expressing clone was isolated from peptide-stimulated PBMCs of patient LAU 169 (LAU 169/4E8); no polyclonal culture was obtained from this patient. Among tetramer$^+$ cells in the polyclonal culture of HD 591, ~22% expressed BV 21.3, whereas 32% expressed BV 22. Among tetramer$^+$ cells in the polyclonal culture of HD 795, ~43% expressed BV 17 and 9% expressed BV 22. Two clones were derived from this culture; one of them (HD 795/4D11) expressed BV 17, whereas BV gene segment usage of the other one (HD 795/2B6) could not be determined by staining with anti-BV antibodies but was determined by PCR (BV 13; Table 2). Finally, tetramer$^+$ cells in the polyclonal culture from patient LAU 42 expressed BV 1 (63%) and BV 13.1 (30%) segments. Interestingly, this last patient expressed the *HLA-A*0206* allele instead of the *HLA-A*0201* allele expressed by the large majority of Caucasians.

The TCR β chain used by MAGE-A10$_{254-262}$-specific clonal populations was analyzed in greater detail by PCR (Table 2). This analysis revealed CDR3 regions of variable size (6–10 amino acids) and sequence among clones suggesting the lack of a high degree of restriction in the repertoire selection of MAGE-A10$_{254-262}$-specific CD8$^+$ T cells. Strikingly, however, clones LAU 203/1B8 and HD 795/2B6, both using BV 13 but different BJ segments, exhibited identical CDR3 sequences (6 amino acids long), with the exception of a single amino acid substitution (in bold in Table 2). Thus, despite the apparent lack of restriction, conserved structural features were also observed among MAGE-A10$_{254-262}$-specific TCRs.

Efficiency and Fine Specificity of T-Cell Recognition of MAGE-A10$_{254-262}$-specific CD8$^+$ T Cells. To assess the efficiency of antigen recognition by MAGE-A10-specific T cells, peptide titration assays were performed for 10 independent T-cell populations in a standard 4-h chromium release assay using the T2 cell line as target cells. An example of the results obtained with this analysis is shown in Fig. 2 for two cell clones. Data obtained for 10 independent populations are summarized in Table 3. Although all MAGE-A10$_{254-262}$-specific T cells were able to efficiently lyse target cells in the presence of saturating concentrations of MAGE-A10$_{254-262}$ peptide, the peptide concentration required to obtain 50% of maximal lysis was highly variable among the different populations ranging from 0.05 nM to >100 nM. Polyclonal cultures and clones isolated from patients LAU 50, LAU 155, LAU 119, and LAU 203 and from donor HD 591 recognized the MAGE-A10 peptide with relatively high efficiency (50% maximal lysis between 0.05 and 0.4 nM) and are thereafter functionally defined as high-avidity T cells. In contrast, clones isolated from donor HD 795 and patients LAU 42 and LAU 169 recognized the Ag with relatively low efficiency (50% maximal lysis at ~100 nM) and are thereafter functionally defined as low-avidity T cells. Both high- and low-avidity-specific T cells were isolated from melanoma patients expressing MAGE-A10 in their tumor cells (LAU 50, LAU 119, and LAU 169; Ref. 19). Similarly, both high- and low-avidity-specific T cells were isolated from patients bearing MAGE-A10-negative tumors as well as from healthy donors (LAU 155, LAU 203, LAU 42, HD 591, and HD 795). Thus, we found no clear correlation between MAGE-A10 expression in their tumor cells and isolation of high *versus* low avidity MAGE-A10-specific T cells.

The fine specificity of recognition of MAGE-A10-specific T-cell populations was evaluated by assessing the recognition of a panel of MAGE-A10$_{254-262}$ single-alanine-substituted peptide analogues in peptide titration experiments. Replacement of Gly at position 1 of the MAGE-A10$_{254-262}$ peptide with Ala resulted in unmodified or increased recognition by a group of clones and highly decreased recognition by a distinct group of clones. Interestingly, all of the clones in the latter group used BV 22. A substitution of Leu at position 2 did not significantly affect T-cell recognition with few exceptions (HD

Table 2 *Sequence analysis of BV transcripts from MAGE-A10-specific T cell clones*

Clone	BV	CDR3β (aa)[a]	CDR3β (nt)	BJ	Occurrence
LAU 42/2D6	BV 1	SLTGYEQ	AGC CTC ACG GGG TAT GAG CAG	BJ 2.1	6/6
LAU 203/1B8	BV 13	SYQGE**A**	AGT TAC CAG GGT GAA GTC	BJ 1.1	5/5
HD 795/2B6	BV 13	SYQGE**Q**	AGT TAC CAG GGT GAG CAG	BJ 2.7	5/5
HD 795/4D11	BV 17	STMGQGDGEL	AGT ACT ATG GGA CAG GGA GAC GGG GAG CTG	BJ 2.2	6/6
LAU 155/6D1	BV 21	SLEYTGEL	AGC TTA GAA TAC ACC GGG GAG CTG	BJ 2.2	5/5
LAU 50/15	BV 21	SFGGLGYEQ	AGC TTT GGG GGG CTG GGC TAC GAG CAG	BJ 2.7	5/5
LAU 169/4E8	BV 22	SEASSYNSPL	AGT GAG GCC AGC TCC TAT AAT TCA CC CTC	BJ 1.6	4/4

aa, amino acid; nt, nucleotide.
[a] Single amino acid substitution is shown in bold.

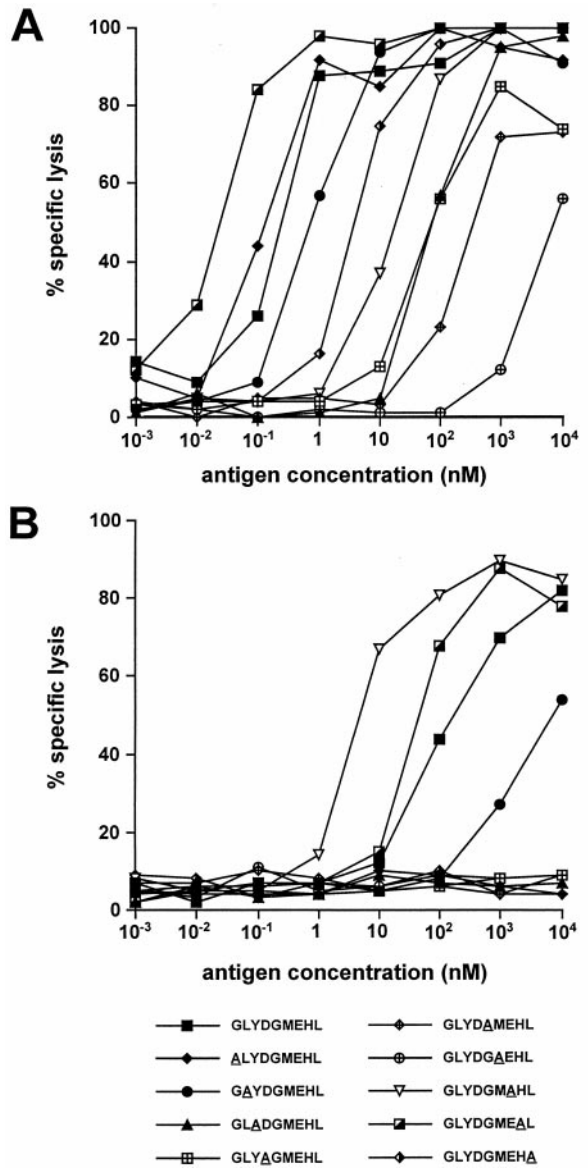

Fig. 2. Avidity and fine specificity of antigen recognition of MAGE-A10$_{254-262}$-specific CD8$^+$ T cells. Avidity and fine specificity of antigen recognition of MAGE-A10$_{254-262}$-specific CD8$^+$ T cells was assessed in a 4-h ^{51}Cr release assay as detailed in "Materials and Methods." An example of the results obtained is shown here for clones LAU 50/15 **(*A*)** and LAU 169/4E8 **(*B*)**. Data obtained for 10 independent populations are summarized in Table 3.

591 P and LAU 203/1B8). Individual substitutions at the remaining amino acid positions had significant negative effect on T-cell recognition for the majority of the clones analyzed, although significant differences in the fine specificity of T-cell recognition were clearly seen among clones.

Correlation between Avidity of Peptide Recognition, Specific Tumor Lysis, and Tetramer Staining. The ability of MAGE-A10-specific CD8$^+$ T cells to specifically lyse the MAGE-A10-expressing tumor cell line Me 275 was assessed by standard chromium release assay (Fig. 3*A* and data not shown). All populations were able to lyse Me 275 in the presence of MAGE-A10$_{254-262}$ peptide. However, only some of them efficiently lysed Me 275 in absence of peptide. Specific tumor lysis tightly correlated with avidity of peptide recognition of MAGE-A10$_{254-262}$-specific populations. As shown in Fig. 3*B*, MAGE-A10$_{254-262}$-specific cells requiring <1 nM of antigenic peptide to achieve 50% maximal lysis on T2 cells were also able to lyse MAGE-A10-expressing tumor cells. In marked contrast, polyclonal cultures or clones that required higher peptide concentrations to achieve 50% maximal lysis failed to do so.

To determine whether the observed differential avidity of the T cells reflected an inherent property of the TCR or whether it was attributable to other factors, such as the level of expression of the TCR or accessory molecules, mAbs to the CD3, TCR$\alpha\beta$, CD8, LFA, and CD28 were used to stain representative CTL clones of different avidity. There were no detectable differences in the levels or TCR expression as well as in the expression of accessory molecules (data not shown). This suggested that the TCR itself was the main determinant imparting the differential avidity of the CTL clones. Hence, a correlation between avidity of CTL clones and the efficacy of tetramer staining could be expected. To address this question, CTL clones of different avidity (Fig. 4*A*) were stained with tetramers during 1 h at room temperature. Under these conditions, two of three high-avidity clones (LAU 50/22 and LAU 155/6D1) displayed a brighter tetramer staining as compared with the three low-avidity clones analyzed (HD 795/2H11, HD 795/4D11, and LAU 168/4E8), whereas one did not (LAU 50/15; Fig. 4*A*). However, interestingly, upon prolonged staining (4 h), high-avidity clones were stained significantly more efficiently than low-avidity clones. Similar results were obtained upon staining of the polyclonal populations from which the clones were derived (Fig. 4*A*). To analyze whether this phenomenon could be exploited to separate high and low tumor-reactive MAGE-A10$_{254-262}$-specific populations, T cells present among polyclonal populations, we performed the experiment illustrated in Fig. 2*B*. Polyclonal populations from HD 795, LAU 50, and LAU 155 (Fig. 4*A*) were mixed in approximately equivalent proportions. The resulting population,

Table 3 *Recognition of substituted MAGE-A10$_{254-262}$ peptide variants by MAGE-A10-specific T cells*

	LAU 50/15 (0.13 nM)[a]	LAU 50/22 (0.21 nM)	LAU 155/6D1 (0.23 nM)	LAU 119/P[b] (0.25 nM)	LAU 203/1B8 (0.05 nM)	LAU 169/4E8 (92 nM)	LAU 42/2D6 (58 nM)	HD 591/P (0.28 nM)	HD 795/2B6 (2 nM)	HD 795/4D11 (42 nM)
Peptide sequence[c]										
GLYDGMEHL	1[d]	1	1	1	1	1	1	1	1	1
ALYDGMEHL	2	0.002	9	<0.0001	2	<0.0001	5	1.2	3	50
GA**A**YDGMEHL	0.4	0.7	2	0.1	0.4	0.01	0.3	0.002	10	1.2
GLA**A**DGMEHL	0.002	0.001	0.005	<0.0001	<0.0001	<0.0001	<0.0001	0.0007	<0.0001	<0.0001
GLYA**A**GMEHL	0.002	0.001	2	<0.0001	0.001	<0.0001	<0.0001	0.0007	<0.0001	<0.0001
GLYDA**A**MEHL	0.0004	0.0001	<0.0001	<0.0001	0.1	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
GLYDGA**A**EHL	0.0001	0.0002	0.001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
GLYDGMA**A**HL	0.01	0.0006	0.004	<0.0001	0.001	8	<0.0001	<0.0001	<0.0001	<0.0001
GLYDGMEA**A**	7	3	2	16	0.0002	1	<0.0001	16	<0.0001	3
GLYDGMEH**A**	0.05	0.006	<0.0001	0.1	0.04	<0.0001	4	0.04	<0.0001	<0.0001

[a] Numbers in parentheses represent the nanomolar concentration of parental MAGE-A10$_{254-262}$ peptide required for 50% maximal lysis by the corresponding T-cell population in a standard 4-h chromium release assay.
[b] P, polyclonal.
[c] Alanine substitutions are in bold.
[d] Relative antigenic activity of single A-substituted MAGE-A10$_{254-262}$ peptide variants as compared with MAGE-A10$_{254-262}$. The relative antigenic activity for each peptide compared with that of the parental peptide was calculated as described in "Materials and Methods."

stained with tetramers during 4 h at room temperature, showed a broad range of tetramer staining so that it could be arbitrarily separated into tetramer bright and tetramer low populations by FACS (Fig. 4*B*). The functional characteristics of tetramer^{high} and tetramer^{low} populations were then analyzed in a chromium release assay in the presence of graded peptide concentrations. Tetramer^{high} T cells exerted 50% maximal lysis at a 100-fold lower peptide dose as compared with tetramer^{low} T cells. In agreement with these data, tetramer^{bright} T cells displayed a considerably higher tumor-specific lysis than tetramer^{low} T cells (Fig. 4*C*). It is of note that the total population displayed a functional activity comparable with that of tetramer^{high} T cells demonstrating that the functional heterogeneity of the unseparated population would have passed unnoticed in a standard analysis.

DISCUSSION

In the present study, we have performed a detailed analysis of MAGE-A10$_{254-262}$ tetramer$^+$ CD8$^+$ T cells derived from both A2$^+$ melanoma patients and healthy donors. The major findings are as follows: (*a*) A2/MAGE-A10$_{256-262}$ tetramer$^+$ cells can use different TCR BV gene segments, suggesting a certain degree of structural diversity; (*b*) the repertoire of these cells is diverse both in terms of clonal composition, efficiency of antigen recognition, and tumor specific lytic activity; (*c*) only cells able to recognize the antigenic peptide with high efficiency are able to lyse MAGE-A10-expressing tumor cells; (*d*) under defined experimental conditions, high- and low-avidity MAGE-A10$_{254-262}$-specific T cells can be separated by FACS.

T-cell Ag recognition is based on the interaction of clonotypic TCR-$\alpha\beta$ with antigenic peptides presented by a given HLA molecule. It is likely that, because of the structural constrains imposed by Ag recognition, a limited number of TCRs exhibiting at least some common features will be selected upon stimulation with Ag, thus defining a limited Ag-specific TCR repertoire. Numerous studies have focused on the complexity of TCR usage by T cells of defined Ag specificity (25). The human TCR repertoire expressed by CTLs specific for MHC class-I restricted viral peptides has been shown to be highly restricted and very similar in different individuals sharing the presenting HLA allele (26–30). Interestingly, overexpression of a restricted set of TCR BV gene families was found in metastatic lesions of melanoma patients treated with an autologous Dinitrophenyl-modified tumor cell vaccine (31). However, the knowledge concerning the repertoire of tumor-specific T cells of defined antigen specificity remains limited. Initial studies on the repertoire of T cells specific for the immunodominant HLA-A2-restricted peptide from the tumor Ag Melan-A$_{26}$ (27–35) gave conflicting results, some reporting restricted (32) and others diverse (21, 33, 34) BV gene segment usage. By using A2/Melan-A peptide tetramers, we have observed recently that Melan-A26 (27–35) specific T cells present in both tumor-infiltrated lymph nodes and tumor-infiltrating lymphocytes of melanoma patients exhibit a large and diverse TCR repertoire. However, dominant clonal populations were also found in individual patients (24, 35).

Because of the rare CTL response to the initially defined MAGE-derived antigenic peptides, the repertoire of T cells specific for MAGE Ag has remained, until the present time, largely unexplored with one exception (36). In that study the analysis of CTL clones from a single melanoma patient and specific for an HLA-A1-restricted epitope derived from MAGE-1 protein suggested that the TCR repertoire directed against this Ag was rather diverse. In the present study, we observed that MAGE-A10$_{254-262}$-specific CD8$^+$ T cells can use at least five different BV segments. Whereas tetramer$^+$ cells frequently used BV 22, BV 22$^+$ tetramer$^+$ T cells could recognize the antigenic peptide with either high or low avidity. The same was observed in the case of BV 13$^+$ tetramer$^+$ T cells. Thus, no strict correlation between BV segment usage and avidity of Ag recognition could be observed. Interestingly, 2 BV 13$^+$ clones (LAU 203/1B8 and HD 795/2B6) recognized the antigenic peptide with high and low avidity, respectively, despite a high degree of similarity in their CDR3 regions. Further studies should unravel an eventual correlation with other structural features of the TCR, such as α chain usage and preferential $\alpha\beta$ TCR chain pairing. In addition, analysis of a larger sample of responding *HLA-A*0201* individuals is needed to substantiate the preferential BV 22 usage by MAGE-A10$_{254-262}$-specific T cells. If so, the propensity of MAGE-A10$_{254-262}$-specific T cells to preferentially use a limited number of BV segments could be exploited for increasing the rate of MAGE-A10$_{254-262}$-specific T-cell detection based on the use of BV-specific, anti-TCR Abs to select and/or preferentially expand these T cells.

It is noteworthy that MAGE-A10$_{254-262}$-specific T cells could be detected and isolated among peptide-stimulated PBMCs from a *HLA-A*0206*-expressing melanoma patient with *HLA-A*0201*/peptide tetramers. Interestingly, for the same patient, CD8$^+$ T-cell responses to at least three additional CTL epitopes [from influenza matrix$_{58-66}$, tyrosinase$_{368-376}$, Melan-A$_{26}$ (27–35), not shown] could also be visualized with *HLA-A*0201*/peptide tetramers. *HLA-A*0201* and *HLA-A*0206* differ at a single position (amino acid 9) of the α-1 domain of the molecule, where Phe is present in *HLA-A*0201* and Tyr in *HLA-A*0201*. It is likely that this difference results in only moderate

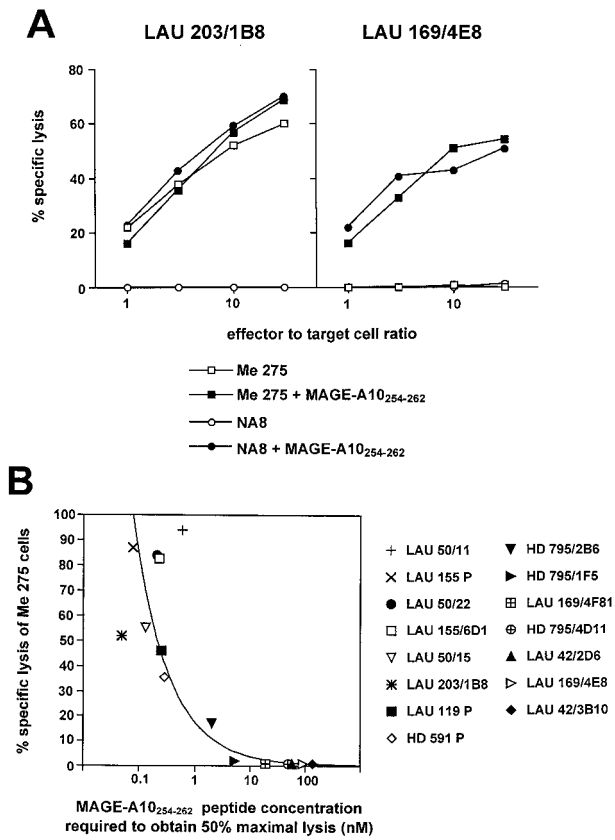

Fig. 3. Correlation between avidity of peptide recognition and specific tumor lysis. *A*, cytolytic activity of CTL clones LAU 203/1B8 and LAU 169/4E8 on melanoma cell lines Me 275 (A2$^+$ MAGE-A10$^+$) and NA8-MEL (A2$^+$ MAGE-A10$^-$) was assessed in a 4-h ^{51}Cr release assay as detailed in "Materials and Methods" in the absence (□) or in the presence (■, ●) of exogenously added peptide MAGE-A10$_{254-262}$ (1 μM). *B*, for each population, the concentration of peptide MAGE-A10$_{254-262}$ required to obtain 50% maximal was plotted against the % specific lysis of MAGE-A10-expressing tumor cell line Me 275.

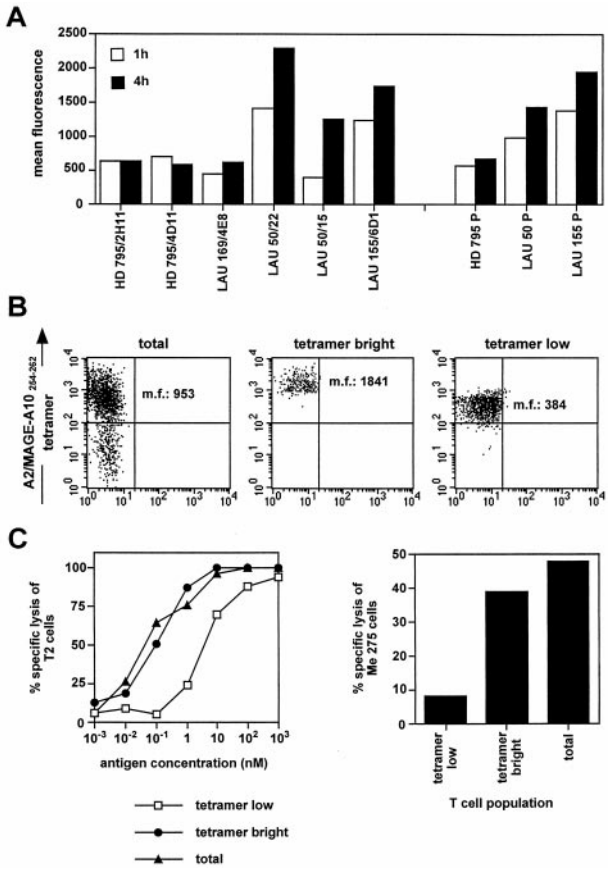

Fig. 4. Correlation between tetramer staining and tumor target recognition. *A*, low-avidity (HD 795/2H11, HD 795/4D11, and LAU 169/4E8) and high-avidity (LAU 50/22 LAU 50/15, and LAU 155/6D1) monoclonal and polyclonal (HD 795 P, LAU 50 P, and LAU 155 P) populations were incubated with A2/MAGE-A10$_{254-262}$ tetramers for 1 h (□) or 4 h (■) at room temperature. After completing the staining, cells were washed and analyzed in a FACSCalibur (Becton Dickinson). Data analysis was performed using the Cell Quest software. *B*, polyclonal populations HD 795 P, LAU 50 P, and LAU 155 P were mixed in equivalent proportions as stained for 4 h with A2/MAGE-A10$_{254-262}$ tetramers at room temperature. After washing, tetramer^{high} and tetramer^{low} populations were sorted by FACS. A2/MAGE-A10$_{254-262}$-specific and nonspecific populations were used as internal controls in the staining. Data acquisition and analysis were performed as in *A*. *C*, cytotoxicity was assessed by ^{51}Cr release assay on T2 cells in the presence of serial dilutions of peptide MAGE-A10$_{254-262}$ or on the melanoma cell lines Me 275 (A2$^+$ MAGE-A10$^+$) in the absence of exogenously added peptide. The lymphocyte:effector T-cell ratio was 10:1.

conformational effects of the corresponding HLA/peptide complexes, thus allowing wide cross-recognition by specific T cells. Nevertheless, MAGE-A10$_{254-262}$-specific T cells from patient LAU 42 were able to recognize the peptide MAGE-A10$_{254-262}$ ~10-fold more efficiently when presented by *HLA-A*0206* than by *HLA-A*0201* (data not shown). The overlapping of CD8$^+$ T cell Ag-specific repertoires restricted to different A2 subtypes is difficult to predict and would be most likely inversely related to the degree of structural conservation among different subtypes. To what extent *HLA-A*0201*/peptide tetramers can be used to analyze peptide-specific CD8$^+$ T-cell responses in individuals expressing other HLA-A2 subtypes is presently unknown. Remarkably, >60% of tetramer$^+$ cells in the polyclonal culture of patient LAU 42 but none in the culture from the seven *HLA-A*0201*-expressing individuals used BV1. Analysis of peptide-stimulated PBMCs from additional *HLA-A*0206*-expressing individuals would be required to determine whether the usage of BV 1 by tetramer$^+$ cells of LAU 42 is indeed related to this particular HLA-A2 subtype.

MAGE-A10$_{254-262}$-specific T cells are functionally heterogeneous both in terms avidity of antigen recognition and of tumor-specific lytic activity. Only MAGE-A10$_{254-262}$-specific CD8$^+$ T cells able to recognize the Ag with high avidity were able to efficiently lyse MAGE-A10-expressing tumors. Similar findings have been reported in the case of CTLs specific for other tumor Ag (13). High avidity of recognition of the tumor target has been shown to be important for antitumor efficacy *in vivo* (14) and could be critical in the case of suboptimal Ag expression (37). One approach for cancer therapy consists in the isolation of tumor-specific T cells from cultures stimulated with peptides corresponding to immunogenic sequences for adoptive transfer into patients. Whereas the isolation and expansion of tumor Ag-specific CTLs can be highly accelerated by tetramer guided cell sorting followed by mitogen driven proliferation (23), it is crucial to identify means of separating high- and low-avidity-specific CTLs from polyclonal populations. In this study, we found a good correlation between intensity of tetramer staining and avidity of T-cell recognition after prolonged incubation with tetramers (4 h) at room temperature. Under these experimental conditions, high and low tumor-reactive MAGE-A10$_{254-262}$-specific T cells could be efficiently separated by FACS. Tetramer guided isolation of high and low tumor-reactive CTLs specific for other tumor Ags from polyclonal cultures has also been reported recently (13). However, the molecular bases of this phenomenon are unclear. Indeed, the intensity of tetramer staining does not appear to be related to differences in the level of expression of the TCR, CD8, or accessory molecules, and it could be the consequence of differences in the association and/or dissociation kinetics of tetramers for individual TCRs. Whereas more rigorous studies would be required to verify these hypotheses, this phenomenon should be taken in consideration in the monitoring of both natural and induced tumor Ag-specific responses.

Additional strategies could be explored to select high avidity MAGE-A10$_{254-262}$-specific T cells. It is noteworthy that in this study, PBMCs were stimulated *in vitro* with a relatively high dose of peptide (1 μM). In previous studies using splenocytes of mice immunized with recombinant viral Ag (38, 39), the dose of antigen appeared to be critical for the optimal elicitation of specific CD8$^+$ T cells and was the function of both T cell avidity and activation state; indeed, if a high dose of Ag induced apoptosis of effector CTLs and optimally activate low avidity CTLs, stimulation with lower doses promoted the expansion of high-avidity CTLs. Whether such approach would be effective for the isolation of high avidity tumor-reactive CTLs from the PBMCs of patients with malignancies remains to be established.

We isolated both high- and low-avidity MAGE-A10$_{254-262}$-specific T cells from PBMCs of healthy donors. In addition, both high- and low-avidity-specific T cells were also isolated from melanoma patients irrespective of MAGE-A10 expression on their melanoma lesions [this study and Valmori *et al.* (19)]. Although these findings suggest the presence of an heterogeneous MAGE-A10$_{254-262}$-specific T-cell repertoire in each individual, they do not exclude the presence, in patients bearing MAGE-A10-expressing lesions, of MAGE-A10$_{254-262}$-specific T cells which would have been spontaneously primed *in vivo* by the tumor. Indeed, a high level of tumor reactivity could be expected by such cells. Unfortunately, the functional phenotype of MAGE-A10$_{254-262}$ tetramer$^+$ T-cell precursors in unstimulated PMBCs cannot be determined because of their low frequency (close or below tetramer detection limits, 1/10,000 CD8$^+$ T cells; Ref. 19). Future studies on peptide-stimulated, purified CD45RA^{bright}/CD45RO^{low} and CD45RA^{low}/CD45RO^{bright} populations (alone or in combination with other markers) will help assess a potential correlation between phenotype and efficiency of tumor recognition by MAGE-A10$_{254-262}$-specific T cells in this patient population, which is eligible for MAGE-A10-based vaccination trials.

The functional heterogeneity of MAGE-A10$_{254-262}$-specific T cells should also be taken in account when considering therapeutic ap-

proaches based on the use of this antigenic peptide for immunization of cancer patients. It can be anticipated that *in vivo* recruitment of high or low tumor-reactive MAGE-$A10_{254-262}$-specific precursors may be dependent on the immunization protocol used. If immunization with MAGE-$A10_{254-262}$ peptide in adjuvant could be expected to recruit only high-avidity T cells because of the low peptide dose, immunization strategies aimed at inducing a high antigen load, such as, *e.g.*, immunization with serum protease-resistant peptides, could also result in the recruitment of low-avidity CTLs. On the other hand, the *in vivo* expansion potential of the two CTL subsets could also be different, because low avidity T cells could be only suboptimally activated by Ag stimulation and therefore be more dependent from help than high-avidity CTLs. Answering these questions may require the establishment of appropriate animal models as well as the accurate monitoring of MAGE-A10 vaccination trials in cancer patients.

ACKNOWLEDGMENTS

We thank Dr. Catherine Servis for peptide synthesis, Nicole Montandon for excellent technical assistance, and Martine van Overloop for assistance in manuscript preparation. We are deeply grateful to the melanoma patients for their generous participation in this research project.

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Heterogeneous T-Cell Response to MAGE-A10$_{254–262}$: High Avidity-specific Cytolytic T Lymphocytes Show Superior Antitumor Activity

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Cancer Res 2001;61:5850-5856.