Generation of CTL Recognizing an HLA-A*0201-Restricted Epitope Shared by MAGE-A1, -A2, -A3, -A4, -A6, -A10, and -A12 Tumor Antigens: Implication in a Broad-Spectrum Tumor Immunotherapy[1]

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MAGE-A1, -A2, -A3, -A4, -A6, -A10, and -A12 are expressed in a significant proportion of primary and metastatic tumors of various histological types and are targets of tumor Ag-specific CTL. Individual MAGE-A expression varies from one tumor type to the other but, overall, the large majority of tumors expresses at least one MAGE-A Ag. Therefore, targeting epitopes shared by all MAGE-A Ags would be of interest in immunotherapy against a broad spectrum of cancers. In the present study, we describe a heteroclitic MAGE-A peptide (p248V9) that induces CTL in vivo in HLA-A*0201 transgenic HHD mice and in vitro in healthy donors. These CTL are able to recognize two low HLA-A*0201 affinity peptides differing at their C-terminal position and derived from MAGE-A2, -A3, -A4, -A6, -A10, and -A12 (p248G9) and MAGE-A1 (p248D9). Interestingly, p248V9-specific CTL respond to endogenous MAGE-A1, -A2, -A3, -A4, -A6, -A10, and -A12 in an HLA-A*0201-restricted manner and recognize human HLA-A*0201$^+$MAGE-A$^+$ tumor cells of various histological origin. Therefore, this heteroclitic peptide may be considered as a potent candidate for a broad-spectrum tumor vaccination. *The Journal of Immunology,* **2002, 169: 575–580.**

The identification of tumor Ags (TA)[3] and their recognition by tumor-specific CTL has fuelled the development of immunotherapeutic strategies in cancer. Although numerous TA and their epitopes have been identified, particularly in melanoma (1, 2), the majority of these are quite restricted in expression and their clinical utility remains limited. Therefore, it is imperative to evaluate additional TA/epitopes that are widely expressed in cancer to develop clinically meaningful immune intervention for most cancer patients regardless of tumor type. The p53 and the recently described human telomerase reverse transcriptase (TERT) are classical examples of widely expressed TA (3, 4). However, the use of these TA for tumor immunotherapy raises two major problems related to their expression by normal tissues, including thymus (5). First, their specific CTL repertoire is composed of low-avidity T cells with weak tumor-killing activity that have escaped tolerance, as demonstrated for p53 (6, 7). Second, targeting widely expressed TA can induce autoimmunity that, in some cases, leads to the destruction of essential normal cells and tissues. We have recently obtained evidence indicating that vaccination with dominant TERT epitopes induces autoimmunity that targets TERT-expressing activated B cells (our unpublished observations).

MAGE-A is a multigene family that consists of 12 homologous genes (MAGE-A1 to -A12) located in the q28 region of chromosome X (8). Among the different family members, MAGE-A1, -A2, -A3, -A4, -A6, -A10, and -A12 are abundantly expressed by tumors but not by normal tissues except testis and placenta (9–23). Each one of the seven MAGE-A Ags is detected in primary and metastatic tumors of various histological types including melanoma, lung, bladder, ovarian, and breast carcinomas. Individual MAGE-A expression varies from one tumor type to the other, but overall the large majority of tumors expresses at least one MAGE-A. Targeting epitopes shared by all MAGE-A Ags would be of interest against a broad spectrum of cancers. Moreover, the use of these epitopes will not come up against the tolerance of their CTL repertoire and the risks of autoimmunity. MAGE-A do not belong to the immunological self and the MAGE-A-expressing testis is considered as an immunoprivileged organ and cannot be the target of an autoimmune response.

To find common MAGE-A epitopes susceptible to induce MAGE-A-specific HLA-A*0201-restricted CTL, we first selected peptides likely to share the same antigenicity, even if they lack the HLA-A*0201-specific anchor residues. Sequence modifications were introduced in some of these peptides to improve their binding capacity and their immunogenicity.

In this work we describe one high-affinity heteroclitic peptide (p248V9) corresponding to two HLA-A*0201-restricted, cross-recognized epitopes, derived from MAGE-A2, -A3, -A4, -A6, -A10, -A12 (p248G9) and MAGE-A1 (p248D9). This heteroclitic peptide stimulates CTL that recognize each MAGE-A Ag individually and kill MAGE-A-expressing tumor cells. Therefore, it may be implemented for a broad-spectrum, efficient, but nonetheless safe, vaccination.

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[3] Abbreviations used in this paper: TA, tumor Ag; TERT, telomerase reverse transcriptase; RA, relative affinity; DC, dendritic cell; DC_{50}, dissociation complex.

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Materials and Methods

Animals

The HLA-A*0201 transgenic HHD mice were previously described (24).

Cells

Murine RMAS/HHD cells were obtained by transfection of murine RMAS cells with the HHD construct as previously described (24). COS-7 cells and WEHI-164 clone 13 cells were kindly provided by Dr. F. Jotereau (Institut National de la Santé et de la Recherche Médicale, Unité 463, Nantes, France). The HLA-A*0201-expressing human tumor cells were T2 (deficient in TAP1 and TAP2 transporters); M44 and M113 (melanoma; kindly provided by Dr. F. Jotereau), OBR (bladder cancer; kindly provided by Dr. D. Zeliszewsli, Centre National de la Recherche Scientifique UPRES-A8067, Paris, France), MCF-7 (breast cancer), and Caco-2 (colon cancer). Cells were grown in RPMI 1640 medium supplemented with 10% FCS.

Peptides and plasmids

Peptides were synthesized by Synt:em (Nîmes, France). Plasmids containing the cDNA for MAGE-A (pcD-Sra/MAGE-A1, pcD-Sra/MAGE-A2, pcDNAI/MAGE-A3, pcDNAI/MAGE-A4, pcDNAI/MAGE-A6, pcDNAI/MAGE-A10, and pcDNAI/MAGE-A12) were kindly provided by Dr. P. van der Bruggen (Ludwig Institute for Cancer Research, Brussels, Belgium). The plasmid containing the HHD construct was described previously (24).

Generation of CTL in HHD mice

HHD mice were injected s.c. with 100 μg of peptide emulsified in IFA in the presence of 140 μg of the I-Ab-restricted HBV core-derived Th epitope (128–140: TPPAYRPPNAPIL). After 11 days, 5×10^7 spleen cells were stimulated in vitro with peptide (10 μM) in RPMI 1640 medium supplemented with 10% FCS, 2 μM glutamine, and antibiotics. On day 6 of culture, the bulk responder populations were tested for specific cytotoxicity. Upon response, CTL cultures were restimulated weekly in vitro with 2×10^7 irradiated spleen cells in the presence of 1 to 0.1 μM peptide and 50 U/ml rIL-2 (Proleukin; Chiron, Suresnes, France).

Generation of CTL from human PBMC

PBMC were collected by leukapheresis from healthy HLA-A*0201 donors. Dendritic cells (DC) were produced from adherent cells cultured for 7 days (2×10^6 cells/ml) in the presence of 500 IU/ml GM-CSF (Leucomax; Schering-Plough, Levallois Perret, France) and 500 IU/ml IL-4 (R&D Systems, Minneapolis, MN) in complete medium (RPMI 1640 supplemented with 10% heat inactivated human AB serum, 2 μM L-glutamine, and antibiotics). On day 7 maturation agents poly(I:C) at 100 ng/ml and anti-CD40 mAb (kindly provided by Dr. J. P. Abastado, Immuno-Designed Molecules, Paris, France) at 2 μg/ml were added in the culture for 24 h. Mature DC were pulsed with 10 μM peptide in the presence of 5 μg/ml β_2-microglobulin for 2 h at 37°C and then irradiated (3500 rad). $CD8^+$ cells were purified by positive selection with CD8 MicroBeads (Miltenyi Biotec, Paris, France) according to the manufacturer's instructions. A total of 2×10^5 $CD8^+$ cells were stimulated with 2×10^4 peptide-pulsed DC in complete culture medium supplemented with 1000 IU/ml IL-6 (R&D Systems) and 5 IU/ml IL-12 (R&D Systems) in a final volume of 100 μl/well in 96-well round-bottom plates. From day 7, cultures were weekly restimulated with peptide-loaded DC in the presence of 20 IU/ml IL-2 and 10 ng/ml IL-7 (R&D Systems). After the third in vitro restimulation, CD8 cells were collected and stimulated with allogeneic HLA-A*0201 EBV-transformed B cells in the presence of 10 μM peptide for 16 h. IFN-γ-producing $CD8^+$ cells were purified using the IFN-γ secretion assay cell enrichment and detection kit (Miltenyi Biotec) according to the manufacturer's instructions. Purified $CD8^+$ cells were then cultivated in complete medium supplemented with 20 IU/ml IL-2 and 10 ng/ml IL-7 for 1 wk.

Cytotoxic assay

Targets were labeled with 100 μCi of ^{51}Cr for 90 min, washed twice, and plated in 96-well round-bottom plates (3×10^3 cells/well in 100 μl of RPMI 1640 plus 5% FCS). When RMAS/HHD cells were used as targets they were pulsed with various concentrations of peptides at 37°C for 90 min. A total of 100 μl of effectors were then added to the wells. After a 4-h incubation, 100 μl of supernatant were collected and radioactivity was measured in a gamma counter. The percentage of specific lysis was determined as follows: lysis = (experimental release − spontaneous release)/(maximal release − spontaneous release) × 100.

Intracellular IFN-γ staining

T cells were stimulated by T2 cells loaded with peptide (10 μM) and by tumor cells in the presence of 20 μg/ml brefeldin A (Sigma, Oakville, Canada). In blocking experiments tumor cells were preincubated with an anti-HLA-A*0201 (BB7.2) and an anti-HLA-B/C (B1.23.2) mAb for 1 h. Six hours later T cells were washed, stained with PE-conjugated anti-CD8 mAb (Caltag Laboratories, Burlingame, CA) in PBS for 25 min at 4°C, washed, and fixed with 4% paraformaldehyde. Then, cells were permeabilized with PBS/0.2% saponin/0.5% BSA (Sigma) and stained with allophycocyanin-conjugated anti-IFN-γ mAb (BD PharMingen, Mississauga, Canada). Cells were analyzed on FACSCalibur (BD Biosciences, Mountain View, CA).

Peptide processing assay on COS-7 transfected cells

A total of 2.2×10^4 simian COS-7 cells were plated in flat-bottom 96-well plates in DMEM 10% FCS, in triplicate per condition. Eighteen hours later, the medium was discarded and 100 ng of each DNA plasmid was put in contact with COS cells in DMEM, 10% Nuserum (Collaborative Biochemical Products, Belford, MA), 10 mM chloroquine, and 10 mg/ml DEAE dextran. After a 4-h incubation at 37°C, transfection medium was discarded and 50 μl PBS 10% DMSO was added for 2 min. Transfected COS-7 cells were incubated in DMEM 10% FCS during 40 h and then used with 5×10^4 murine CTL in a TNF-α secretion assay.

TNF-α secretion assay

Transfected COS-7 cells at day 4 and human tumor cells were used as stimulating cells. When necessary, they were incubated with 10 μM peptide for 2 h. A total of 5×10^4 T cells were then added in 50 μl RPMI and 10% FCS and incubated for 6 h. Each condition was tested in triplicate. A total of 50 μl of the supernatant was collected. Standard dilutions were prepared in 50 μl with final doses of TNF-α ranging from 10^4 to 0 pg/ml. On both the supernatants and the standard dilutions, 3×10^4 TNF-α sensitive WEHI-164c13 cells in 50 μl were added. They were incubated for 16 h at 37°C. Inhibition of cell proliferation was evaluated by the MTT colorimetric method (25).

MAGE-A expression by human tumor cells

Isolation of total RNA from tumor cells was performed using TRIzol (Invitrogen, Cergy Pontoise, France) reagent. For cDNA synthesis, RNA (2 μg) was diluted in water, 4 μl of 5× reverse transcriptase buffer (Invitrogen), 1 μl of 10 mM dNTP, 2 μl of a 20 μM solution of oligo(dT), 20 U of RNasin (Promega, Madison, WI), 2 μl of 0.1 M DTT, and 200 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen) in a 20-μl reaction volume, and incubated at 42°C for 60 min. PCR amplification was performed with DNA polymerase TaKaRa *Taq* (Takara Biomedicals, Shiga, Japan) using pairs of oligonucleotide primers that are highly specific for each MAGE-A gene (8, 26–28). To ensure that the RNA is not degraded, a PCR assay with primers specific for β-actin was conducted. Samples were scored positive when a band of appropriate size was visible on an agarose gel in the presence of ethidium bromide.

*Measurement of peptide RA to HLA-A*0201*

The protocol used has been described previously (29). Briefly, T2 cells were incubated with various concentrations of peptides ranging from 100 to 0.1 μM at 37°C for 16 h, and then stained with the BB7.2 mAb to quantify the expression of HLA-A*0201. For each peptide concentration, the HLA-A*0201-specific staining was calculated as the percentage of staining obtained with 100 μM of the reference peptide HIVpol 589 (IVGAETFYV). The relative affinity (RA) was determined as follows: RA = (concentration of each peptide that induces 20% of HLA-A*0201 expression/concentration of the reference peptide that induces 20% of HLA-A*0201 expression).

*Assessment of peptide/HLA-A*0201 complex stability*

As previously described (29), T2 cells were incubated overnight with 100 μM of each peptide at 37°C in serum-free medium. Cells were washed, incubated with brefeldin A (10 μg/ml) for 1 h, washed again, and incubated at 37°C during 0, 2, 4, or 6 h in the presence of brefeldin A at 0.5 μg/ml. Cells were stained with the BB7.2 mAb. Dissociation complex (DC_{50}) was defined as the time required for the loss of 50% of the HLA-A*0201/peptide complexes stabilized at $t = 0$ h.

Table I. *Frequency of MAGE-A expression by human cancers*

Cancer	Frequency of Expression (%)							Reference
	MAGE-A1	MAGE-A2	MAGE-A3	MAGE-A4	MAGE-A6	MAGE-A10	MAGE-A12	
Melanoma	16	E[a]	36	E	64	E	74	10, 11
Head and neck	25	42	33	8	N[b]	N	N	16
Bladder	21	30	35	33	15	N	9	12
Breast	6	19	10	13	5	N	N	13
Colorectal	N	5	5	N	5	N	N	17, 23
Lung	21	30	46	11	8	N	N	15, 22
Gastric	30	22	57	N	N	N	N	9
Ovarian	55	32	20	E	20	N	N	19, 21
Osteosarcoma	62	75	62	12	62	N	N	14
Hepatocarcinoma	68	30	68	N	30	30	30	20
RCC[c]	22	16	76	30	N	N	N	18

[a] E, Expressed but the frequency is not known.
[b] N, Expression by tumors has never been studied.
[c] MAGE-A expression in renal cell carcinoma (RCC) is not confirmed in other studies (11).

Results

Selection of peptides belonging to different MAGE-A Ags and sharing the same antigenicity

The MAGE-A1, -A2, -A3, -A4, -A6, -A10, and -A12 are expressed in a large variety of tumors with a frequency ranging from 5 to 76% (Table I). However, a very high percentage of tumors express at least one MAGE-A.

The aim of this work was to identify epitopes belonging to different MAGE-A and sharing the same antigenicity. The antigenicity of HLA-A*0201-bound peptides is defined by their sequence that interacts with the TCR and is located between the P3 and P8/9. We aligned the sequences of MAGE-A1, -A2, -A3, -A4, -A6, -A10, and -A12 to find nonamers and decamers sharing the P3–P8 and P3–P9 regions, respectively. Four groups of peptides were identified, and their sequences and the MAGE-A they belong to are shown in Table II. Peptides were designed by the position of the first residue in the MAGE-A1 sequence. p262 had a complete sequence homology in all MAGE-A, whereas p174 was identical in all MAGE-A Ags except MAGE-A10. The homology group p248 was composed by two peptides, p248G9 (MAGE-A2, -A3, -A4, -A6, -A10, and -A12) and p248D9 (MAGE-A1), differing at the C-terminal position. The fourth group (p264) was excluded from this study because it corresponds to the already known HLA-A*0201-restricted MAGE-A3 epitope (FLWGPRALV) that is reported to be not efficiently processed by tumor cells (30).

*Affinity of selected peptides for HLA-A*0201*

We then evaluated the capacity of selected peptides to bind (RA) and to stabilize (DC_{50}) the HLA-A*0201 molecule (Table II). As we reported previously, these parameters allow for distinguishing high-affinity peptides (RA $<$ 5 and $DC_{50} > 2$ h) that are immunogenic in both HHD mice and humans from low-affinity peptides (RA $>$ 5 and $DC_{50} < 2$ h) that are nonimmunogenic (29). According to these criteria, peptides p174, p262, p248G9, and p248D9 had a low affinity for HLA-A*0201. To enhance affinity of p174, p262, p248G9, and p248D9 and thus increase their immunogenicity, we modified their sequences at HLA-A*0201-specific primary (P2 and P9/10) and/or secondary anchor positions. p262, p248G9, and p248D9 lacked the anchor residues at P2 or C-terminal position. The heteroclitic variants p262L2 and p248V9 exhibited high binding affinity (RA = 0.2 and 1.8, respectively) and stabilization capacity (DC_{50} = 6 and 4 h, respectively). For the p174 we produced the p174Y1V10 variant by replacing the residue at P1 with a tyrosine as previously described (29) and the residue at P10 with the strongest anchor valine. P174Y1V10 was a strong binder (RA = 2.5) but weakly stabilized the HLA-A*0201 molecule ($DC_{50} < 2$ h).

Generation of native peptide-specific CTL by vaccination with heteroclitic variants

To study whether heteroclitic peptides can induce CTL able to recognize the corresponding native peptides, we vaccinated HHD mice with heteroclitic peptides, stimulated their spleen cells with 10 μM peptide in vitro, and tested cultures for killing RMAS/HHD cells loaded with the heteroclitic or the native peptides (Fig. 1*A*). p174Y1V10 was unable to elicit a CTL response, probably because of its weak stabilization capacity. CTL generated in p262L2-vaccinated mice recognized the heteroclitic variant but not the native p262. In contrast, p248V9-vaccinated mice developed a CTL response directed against the heteroclitic but also against the native

Table II. *RA and HLA-A*0201 stabilization capacity (DC_{50}) of peptides*

Peptides	Sequence	MAGE-A	RA[a]	DC_{50}[b]
p174	CLGLSYDGLL	1, 2, 3, 4, 6, 12	41	<2
p174Y	YLGLSYDGLL		13	<2
p174Y1V10	YLGLSYDGLV		2.5	<2
p248G9	YLEYRQVPG	2, 3, 4, 6, 10, 12	>27	<2
p248D9	YLEYRQVPD	1	22.5	<2
p248V9	YLEYRQVPV		1.8	4
p262	YEFLWGPRA	1, 2, 3, 4, 6, 10, 12	>35	<2
p262L	YLFLWGPRA		0.2	6

[a] RA = concentration of each peptide/concentration of the reference peptide that induce 20% of HLA-A*0201 expression obtained by 100 μM of the reference peptide.
[b] Half-life of the peptide/HLA-A*0201 complex.

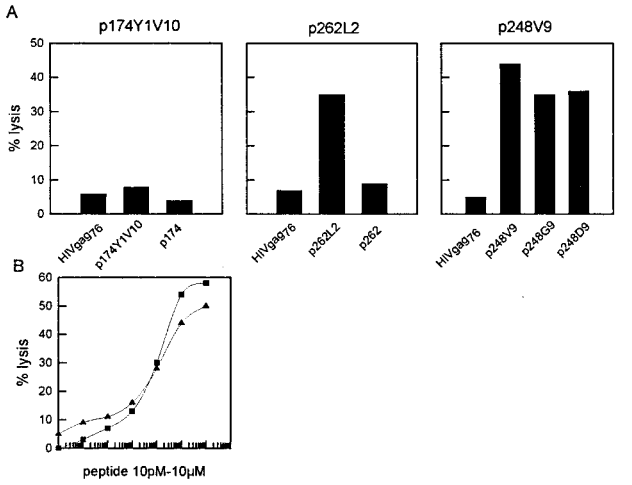

FIGURE 1. Immunogenicity of heteroclitic common MAGE-A peptides. *A*, HHD mice were vaccinated with heteroclitic peptides as indicated in *Materials and Methods* and their spleen cells were restimulated in vitro with 10 μM of corresponding peptides. CTL activity was tested 6 days later on RMAS/HHD targets pulsed with the irrelevant HIVgag$_{76}$, the heteroclitic and the corresponding native peptide. The E:T ratio was 40:1. At least six mice were tested for each peptide and representative results from one mouse are presented. *B*, CTL248 line established as described in *Materials and Methods* was tested against RMAS/HHD cells preincubated with various concentrations of the native p248G9 (■) and p248D9 (▲) peptides. Lysis of RMAS/HHD cells loaded with the irrelevant HIVgag$_{76}$ peptide (10 μM) was 3%. The E:T ratio was 10:1.

p248G9 and p248D9 peptides. We established a CTL line (CTL248) from HHD spleen cells primed in vivo with p248V9 and in vitro repetitively restimulated with decreasing concentrations of p248G9 (10 to 1 μM). CTL248 was maintained in culture with 1 μM p248G9 and recognized the p248G9 and p248D9 with a relatively high avidity (Fig. 1*B*).

CTL248 cell line recognizes endogenously processed MAGE-A Ags

To test whether p248V9-induced CTL recognize endogenously processed MAGE-A1, -A2, -A3, -A4, -A6, -A10, and -A12, we first stimulated the CTL248 line with COS cells cotransfected with cDNA encoding the HHD construct (α_1 and α_2 domains of HLA-A*0201 and the α_3 and intracellular domains of D^b) and each one of the seven MAGE-A. CTL activation was evaluated by measure of TNF-α secretion. Results show that the CTL248 line responded to COS cells coexpressing HHD and any of the MAGE-A tested but not to COS cells expressing HHD or MAGE-A separately (Fig. 2). This demonstrates that CTL248 recognize all the endogenous MAGE-A presented by HLA-A*0201.

In a second set of experiments, we tested whether CTL248 recognize HLA-A*0201 and MAGE-A-expressing human tumor cells. We stimulated CTL248 with the M44 (MAGE-A1, -A2, -A3, and -A4), M113 (MAGE-A2 and -A3), OBR (MAGE-A6 and -A10), and the MAGE-A-negative MCF-7 and Caco-2 tumor cell lines. All these lines were HLA-A*0201 positive and the MAGE-A expression was assessed by RT-PCR. CTL activation was evaluated by measure of secreted TNF-α. CTL248 responded to all the MAGE-A^+ cells but not to MCF-7 and Caco-2 cells (Fig. 3). These results were further confirmed by measuring the number of IFN-γ-producing CTL248 cells upon stimulation (data not shown). To demonstrate that tumor cell recognition was HLA-A*0201 restricted, we stimulated CTL248 with M44 cells in the presence of an anti-HLA-A*0201 (BB7.2) and an anti-HLA-B/C

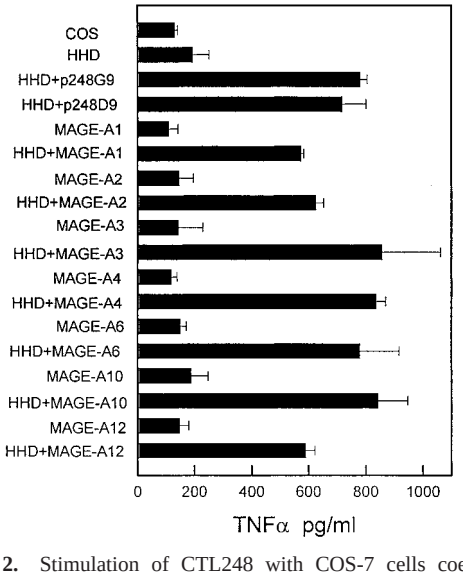

FIGURE 2. Stimulation of CTL248 with COS-7 cells coexpressing HHD and each one of the MAGE-A. CTL248 cells were stimulated with COS-7 cells expressing HHD and each one of the MAGE-A as indicated. In positive control groups CTL248 cells were stimulated with HHD-expressing COS-7 cells incubated with the native peptides (10 μM). CTL248 activation was evaluated by measuring secreted TNF-α using the TNF-α-sensitive WEHI cell line. Results represent the mean ± SD of triplicates. Results for each MAGE-A were confirmed in at least three independent experiments.

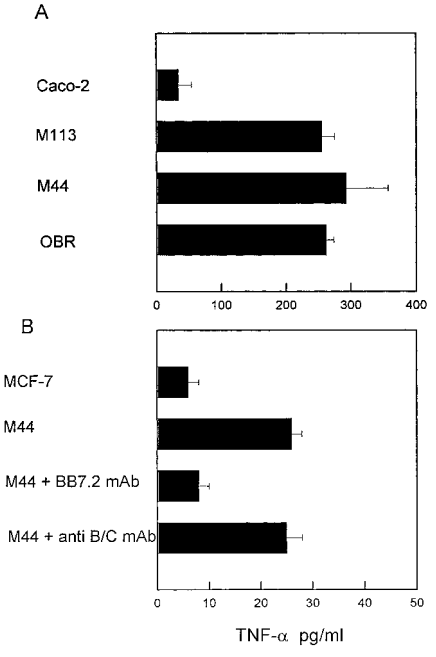

FIGURE 3. Stimulation of CTL248 by MAGE-A-expressing human tumor cells. *A*, CTL248 cells were stimulated with the HLA-A*0201$^+$ MAGE-A^+ M44 (MAGE-A1, -A2, -A3, and -A4), M113 (MAGE-A2 and -A3), OBR (MAGE-A6 and -10), and HLA-A*0201$^+$MAGE-A^- Caco-2 tumor cell lines. CTL248 activation was evaluated by measure of TNF-α secretion. These results were confirmed in two independent experiments. *B*, CTL248 cells were stimulated with M44 tumor cells in the presence of the anti-HLA-A*0201 (BB7.2) or the anti-HLA-B/C (B1.23.2) mAbs. CTL248 activation was evaluated by measure of TNF-α secretion. Data are representative of results of two experiments.

(B1.23.2) mAb. CTL248 response was inhibited by the BB7.2 but not by the B1.23.2 mAb (Fig. 3*B*).

Taken together these data show that p248V9-induced CTL recognize endogenously processed MAGE-A1, -A2, -A3, -A4, -A6, -A10, and -A12.

P248V9 stimulates CTL from healthy donors' PBMC

We then assessed the capacity of p248V9 to generate CTL in vitro from HLA-A*0201 healthy donors' PBMC. CTL were induced by three weekly in vitro stimulations with the p248V9. IFN-γ-producing cells upon peptide stimulation were isolated and cultured for 7–10 days before testing. CTL were generated in seven of nine healthy donors tested. Results from one donor are presented in Fig. 4. CTL responded to T2 cells loaded with p248V9, p248G9, and p248D9 but not to T2 cells loaded with an irrelevant peptide (HIV-gag_{76}) (Fig. 4*A*). They responded also to HLA-A*0201$^+$MAGE-A$^+$ M44 and M113 but not to the HLA-A*0201$^+$MAGE-A$^-$ Caco-2 cells (Fig. 4*B*). We verified the HLA-A*0201 restriction of M44 cell recognition in an anti-HLA-A*0201 Ab blocking experiment. The response of CTL to M44 cells was blocked by the HLA-A*0201-specific BB7.2 Ab but not by the HLA-B/C-specific B1.23.2 Ab (Fig. 4*C*).

These results demonstrate that the heteroclitic p248V9 stimulates human CTL that recognize MAGE-A-expressing tumor cells.

Discussion

In this paper we describe a heteroclitic peptide (p248V9) that stimulates CTL able to recognize endogenous MAGE-A1, -A2, -A3, -A4, -A6, -A10, and -A12 Ags presented by HLA-A*0201. The p248V9 peptide corresponds to two new, low HLA-A*0201 affinity, cross-recognized cryptic epitopes derived from MAGE-A2, -A3, -A4, -A6, -A10, and -A12 (p248G9) and MAGE-A1 (p248D9). This heteroclitic epitope may have a great interest for tumor immunotherapy for several reasons. First, it may generate CTL directed against a very large variety of tumors. In fact, although MAGE-A Ags are expressed by different tumors with a frequency ranging between 5 and 75% (Table I), >80% of all tumors express at least one MAGE-A Ag and could, therefore, be targeted by the p248V9-induced CTL. Second, the use of p248V9 peptide for tumor immunotherapy will lower the risk of tumor escape due to loss of nominal Ag (31, 32). Considering that very often tumors simultaneously express several MAGE-A Ags (33), to escape p248V9-specific CTL response these tumors will have to lose all the MAGE-A they express, which is less likely. Third, because MAGE-A is not expressed in normal tissues except testis, the p248G9- and p248D9-specific CTL repertoire recruited by the heteroclitic p248V9 epitope should not have been negatively selected and the high-avidity CTL providing the strongest antitumor effect (34) should not have been eliminated. Fourth, the absence of MAGE-A in normal tissues limits the risk of autoimmunity that could be a great problem for other universal TA, like the human TERT, which is expressed in activated T and B cells and in the CD34$^+$ hematopoietic progenitors (5). The three latter points underline the advantage of targeting the p248G9 and p248D9 MAGE-A epitope compared with targeting epitopes from other universal TA like human TERT or p53.

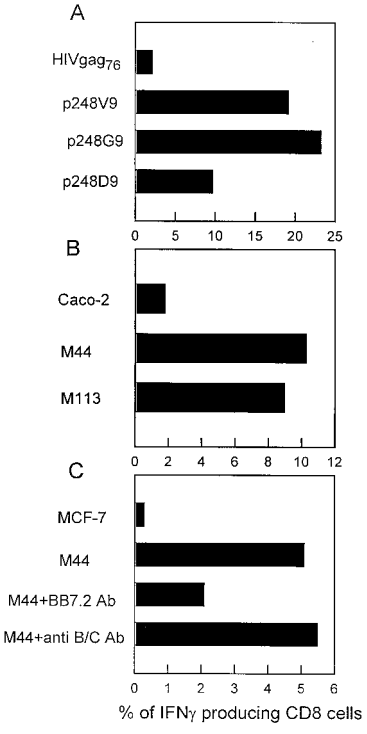

FIGURE 4. Generation of human CTL that recognize MAGE-A-expressing tumor cell lines. CD8$^+$ cells from healthy donors' PBMC were in vitro stimulated with heteroclitic p248V9 peptide-loaded autologous DCs three times at 1-wk intervals. After the third stimulation p248V9-specific CTL were purified as described in *Materials and Methods*, cultured for an additional week, and tested for recognition of native peptide-loaded T2 cells (*A*) and HLA-A*0201$^+$MAGE-A$^+$ tumor cell lines (*B* and *C*). In blocking experiments (*C*), tumor cells were preincubated with BB7.2 and B1.23.2 mAbs. CTL activation was evaluated by measure of IFN-γ-producing cells as assessed by intracellular IFN-γ staining. Data are representative of results of two experiments.

The homology between the different MAGE-A varies from 48% (MAGE-A10 vs MAGE-A12) to 95% (MAGE-A3 vs MAGE-A6). As a result, the alignment of all the MAGE-A sequences reveals only one completely identical nonameric peptide (p262). However, the complete homology is not necessary for peptides to be cross-recognized by CTL. The homology can be limited to the central segment of peptides (P3–P8/9) that contains residues contacting the TCR (35). Moreover, the presence of the HLA-A*0201-specific primary anchor motifs, which is a prerequisite for a high affinity, and immunogenicity were not necessary either. The p248G9 and p248D9 epitopes did not have an anchor motif at P9 in any of the MAGE-A sequences and exhibited a low affinity for HLA-A*0201. The substitution of the P9 residue with the anchor motif V gave an heteroclitic peptide with high affinity, strong immunogenicity, and the same antigenicity as the native peptides. The preservation of the native peptide antigenicity has been reported for several heteroclitic peptides, having substituted the anchor residues (36–39). The rare exceptions of this rule must concern very nonconservative substitutions, as is the case for the p262L2, where an E is substituted by an L at P2. In conclusion, the search of cross-recognized HLA-A*0201-restricted peptides from sequences with relatively high homology can be exclusively based on their homology in the P3/C-terminal P segment. This strategy can effectively be applied to other multigenic families of TA like HER, BAGE, and GAGE to enrich the pool of universal tumor epitopes. The search for a common HER epitope is currently under way in our laboratory.

The p248G9/D9 is the unique common MAGE-A epitope presented by HLA-A*0201. The p262, which is identical to all MAGE-A sequences, is unlikely to be naturally processed because of the unfavorable residue (E) at the anchor P2. Moreover, the previously described MAGE-A3 epitope FLWGPRALV, which differs in the seven MAGE-A only at the C-terminal position, has recently been shown to be ineffectively processed by tumor cells (30). Concerning the numerous other HLA-A*0201-restricted MAGE-A epitopes, their sequences are either unique to the

MAGE-A they belong to or they are shared by very few MAGE-A (40–42). One of the rare exceptions is the HLA-B*3701-restricted promiscuous epitope described by Tanzarella et al. (23). This epitope is shared by MAGE-A1, -A2, -A3, and -A6 but not by MAGE-A4, -A10, and -A12.

In conclusion, in this paper we describe an MAGE-A-derived HLA-A*0201-restricted heteroclitic peptide (p248V9) that induces CTL able to recognize each MAGE-A Ag individually and subsequently to kill MAGE-A-expressing tumor cells. This heteroclitic peptide can be used for a broad-spectrum tumor immunotherapy.

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