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A peptide encoded by the human *MAGE3* gene and presented by HLA-B44 induces cytolytic T lymphocytes that recognize tumor cells expressing *MAGE3*

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Abstract The human *MAGE3* gene is expressed in a significant proportion of tumors of various histological types, but is silent in normal adult tissues other than testis and placenta. Antigens encoded by *MAGE3* may therefore be useful targets for specific antitumor immunization. Two antigenic peptides encoded by the *MAGE3* gene have been reported previously. One is presented to cytolytic T lymphocytes (CTL) by HLA-A1, the other by HLA-A2 molecules. Here we show that *MAGE3* also codes for a peptide that is presented to CTL by HLA-B44. *MAGE3* peptides containing the HLA-B44 peptide binding motif were synthesized. Peptide MEVDPIGHLY, which showed the strongest binding to HLA-B44, was used to stimulate blood T lymphocytes from normal HLA-B44 donors. CTL clones were obtained that recognized not only HLA-B44 cells sensitized with the peptide, but also HLA-B44 tumor cell lines expressing *MAGE3*. The proportion of metastatic melanomas expressing the *MAGE3*/HLA-B44 antigen should amount to approximately 17% in the Caucasian population, since 24% of individuals carry the *HLA-B44* allele and 76% of these tumors express *MAGE3*.

Introduction

Cytolytic T lymphocytes (CTL) directed against human tumor cells can be obtained by the culture of blood lymphocytes of cancer patients with irradiated autologous tumor cells (Boon et al. 1994). Anti-tumor CTL clones can then be derived from the responder T-cell populations. Several genes coding for antigens recognized by such CTL clones have been identified. The *MAGE1* gene codes for an antigenic peptide, MAGE1.A1, recognized on HLA-A1 by autologous CTL directed against a melanoma cell line derived from patient MZ2 (Traversari et al. 1992; van der Bruggen et al. 1991). *MAGE1* belongs to a family of closely related genes clustered within two distinct regions on chromosome X. *MAGE1* to -12 are located in the *Xq26* region, while an additional member has been identified in the *Xp21.3* region (De Plaen et al. 1994; Muscatelli et al. 1995). The *MAGE* gene family is silent in normal adult tissues, with the exception of testis and placenta (De Plaen et al. 1994). *MAGE1*, 2, 3, 4, 6, and 12 are expressed in a significant proportion of tumors of various histological types, including melanomas, lung tumors, head and neck tumors, sarcomas, and breast tumors (Brasseur et al. 1992; Brasseur et al. 1995; De Plaen et al. 1994; Weynants et al. 1994). The sequences of the proteins encoded by *MAGE2*, 3, 4, 6 and 12 display 57% to 77% identity to that of *MAGE1* (De Plaen et al. 1994). The function of these proteins is unknown at present. The *MAGE3* gene, which is expressed by 76% of metastatic melanoma samples (Brasseur et al. 1995), was found to code for another peptide, MAGE3.A1, also presented by HLA-A1 and recognized by anti-tumor CTL of patient MZ2 (Gaugler et al. 1994).

Because the proteins encoded by *MAGE* genes contain more than 300 amino acids, they are expected to contain several peptides that can associate with various HLA molecules and hence may provide many potential target antigens for specific anti-tumor T-cell responses. One method of identifying these new tumor antigens starts with the examination of *MAGE* sequences for known

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HLA-binding peptide motifs. Potentially relevant peptides deduced from the gene sequence can then be synthesized and tested for their ability to bind to the relevant HLA molecule. The best binders are used to stimulate T cells in vitro in order to obtain CTL that recognize the relevant peptide-HLA complexes. It should then be ascertained that these CTL recognize tumor cells expressing the relevant *HLA* and *MAGE* gene. With lymphocytes from normal individuals, this strategy led to an independent identification of the *MAGE3.A1* antigenic peptide (Celis et al. 1994), and more recently to the identification of a *MAGE3* antigen presented by HLA-A2 (van der Bruggen et al. 1994). Here we report that by using this strategy we were able to derive HLA-B44-restricted anti-tumor CTL that recognize a *MAGE3* peptide.

Materials and methods

Cell lines

Tumor cell lines and Epstein-Barr virus (EBV)-transformed B cell lines were cultured in Iscove's medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), L-arginine (116 mg/L), L-asparagine (36 mg/L), and L-glutamine (216 mg/L). Cells of the MHC class I-negative lymphoblastoid cell line C1R (Storkus et al. 1987), transfected either with a cDNA clone encoding *HLA-B*4402* or with an *HLA-B*4403* genomic clone (Fleischhauer et al. 1994; Fleischhauer et al. 1990), were grown in RPMI-1640 medium (Gibco) supplemented with 10% FCS.

In contrast to all the other EBV-transformed B cells that we tested, C1R cells express the *MAGE3* gene. The C1R-B44 transfectants were lysed by the anti-*MAGE3*B44 CTL clones described below.

Synthetic peptides

Peptides were synthesized on solid phase using Fmoc for transient NH₂-terminal protection as described by Atherton and co-workers (1981) and were characterized by mass spectrometry. All peptides were >90% pure as indicated by analytical HPLC. Lyophilized peptides were dissolved in DMSO and diluted in either PBS or acetic acid (10 mM), and stored at -80 °C.

Cytokines

Human recombinant IL-2 and IL-4 were donated by Biogen (Geneva, Switzerland) and R. Devos (Roche Research Center, Gent, Belgium), respectively. One unit/ml of IL-2 gives 50% maximal proliferation of CTLL-2 cells. One unit/ml of IL-4 gives 50% maximal proliferation of human T cells previously activated with phytohemagglutinin A. Human recombinant IL-6 was obtained from J. Van Snick (our laboratory). One unit/ml of IL-6 gives 50% maximal proliferation of 7TD1 cells. Human recombinant IL-7 was purchased from R&D Systems Europe (Abingdon, Oxon, U. K.). Human recombinant IL-12 (specific activity: 5.26×10^6 units/mg) was donated by S. Wolf (Genetics Institute, Cambridge, Massachusetts, USA). Human recombinant GM-CSF was purchased from Sandoz (Sandoz Pharma, Basel, Switzerland).

Competition assay

The C1R-B*4402 or C1R-B*4403 cells were ⁵¹chromium-labeled for 1 h at 37 °C in the presence of human-specific class I MHC monoclonal antibody (mAb) W6/32 (30% [v/v] of culture medium of the hybridoma cells) and washed three times. The labeled cells (1000 cells

in 80 µl) were incubated in V-bottom microwells for 30 min at 20 °C in serum-free medium X-VIVO 10 (Wittaker Bioproducts, Walkersville, MD) with various concentrations of competitor peptides. Antigenic peptide EEKLIVVAF was then added in X-VIVO 10 medium (40 µl) at a final concentration of 50 ng/ml for C1R-B*4402 cells and of 160 ng/ml for C1R-B*4403 cells. Cells were incubated for 30 min at 20 °C and washed in Iscove's medium containing 2% FCS. CTL clone LB33-CTL-159/5 (20000 cells in 150 µl; Coulie et al. 1995), which recognizes peptide EEKLIVVAF in the context of both HLA-B*4402 and HLA-B*4403 targets, was added in Iscove's medium supplemented with 10% human serum. Chromium release was measured after 3 h. Percentage control lysis was calculated as $100 \times [(\% \text{ lysis with competitor-background lysis}) / (\% \text{ lysis without competitor-background lysis})]$. Background lysis is the % lysis of target cells in the absence of peptides and was 1% and 3% for C1R-B*4402 and C1R-B*4403, respectively. Lysis without competitor peptides was 58% for C1R-B*4402 and 72% for C1R-B*4403. Peptide EEKLIVVAF is a variant of peptide EEKLIVVLF which is presented to CTL 159/5 by the melanoma cells of patient LB33 (Coulie et al. 1995). The variant peptide was better recognized on HLA-B*4403 than the original peptide. No difference was observed on HLA-B*4402.

Induction of CTL

Peripheral blood mononuclear cells (PBMC) were isolated using Lymphoprep (Nycomed Pharma, Oslo, Norway) density gradient centrifugation. T lymphocytes were purified by rosetting with 2-aminoethylisothiouonium bromide hydrobromide-treated sheep erythrocytes (Mikamo 1988), and labeled with an CD8-specific mAb coupled to magnetic microbeads (Miltenyi Biotec, Germany). CD8⁺ cells were sorted by passage through a magnet and subsequently frozen. The non-rosetting PBMC were left to adhere for 2 h at 37 °C on Tissue Culture wells (NUNC Multidish 6, Roskilde, Denmark). Non-adherent cells were discarded, and adherent cells were cultured for 7 days in the presence of IL-4 (50 units/ml) and GM-CSF (100 ng/ml) in RPMI-1640 medium + 10% FCS. The resulting cell populations were analyzed for the expression of CD11c and CD14 surface antigens, using flow cytometry. For donor LB816 most of the cells were CD11c⁺ CD14⁻, whereas for donor LB822 most of them were CD11c⁺ CD14⁻. These antigen-presenting cells (5×10^5 to 10^6) were incubated in 2 ml wells (NUNC) for 4 h at 37 °C in 400 µl Iscove's medium supplemented with human β_2 -microglobulin (β_2 m, 2.5 µg/ml, Sigma) and peptide MEVDPIGHLY (50 µg/ml). The peptide-pulsed cells were then irradiated at 50 Gy and washed. Autologous CD8⁺ T cells (2×10^6) in culture medium [(Iscove's medium + 10% human serum, L-arginine, L-asparagine, L-glutamine, and 2-mercaptoethanol (0,05 mM)] supplemented with IL-6 (1000 units/ml) and IL-12 (5 ng/ml) were added. On day 7, the lymphocytes were restimulated with adherent autologous PBMC pulsed with the peptide. PBMC (5×10^6) were left to adhere for 2 h at 37 °C in 400 µl Iscove's medium containing β_2 m and peptide MEVDPIGHLY as before. Peptide-pulsed adherent cells were irradiated and washed. Responder lymphocytes in culture medium supplemented with IL-2 (10 units/ml) and IL-7 (5 ng/ml) were added. On day 14, lymphocytes were restimulated with autologous PBMC pulsed with the peptide. PBMC (2×10^7 /ml) were incubated for 2 h at 20 °C in Iscove's medium with β_2 m and peptide MEVDPIGHLY as before, irradiated, washed, and resuspended at 2×10^6 /ml in culture medium supplemented with IL-2 and IL-7 as before. These stimulator cells (2×10^6) were added to each well containing the responder cells.

On day 21, lymphocytes from cultures showing peptide-specific lytic activity were cloned by limiting dilution in culture medium supplemented with IL-2 (50 units/ml) and IL-4 (5 units/ml). LB816 clones were stimulated with irradiated C1R-B*4402 cells (150 Gy, 8000 cells/well) that had been incubated for 1 h at 20 °C with peptide MEVDPIGHLY (1 µg/ml) and washed. LB822 clones were stimulated with irradiated MZ2-MEL tumor cells (100 Gy, HLA-B*4403, MAGE-3⁺, 8000 cells/well). Irradiated allogeneic LG2-EBV cells (100 Gy, 20000 cells/well) were added as feeder cells to the clones. The microcultures were likewise stimulated each week. After 4 weeks, CTL clones LB816-340/1 and LB822-346/8 were stimulated each week in 2 ml wells with 2×10^5 irradiated C1R-B*4402 or C1R-B*4403 cells that had been incubated for 1 h at 20 °C with peptide MEVDPIGHLY

(1 µg/ml) and washed, and with 10⁶ irradiated LG2-EBV cells, all in culture medium supplemented with IL-2 and IL-4 as before.

Transfection and CTL stimulation assay

COS-7 cells (15000/well) were co-transfected, using the DEAE-dextran-chloroquine method (Seed and Aruffo 1987), with 50 ng of plasmid pcDNA1/Amp containing the cDNA of *MAGE3* and either 50 ng of plasmid pcDNA1/Amp containing the cDNA of HLA-B*4403 or 50 ng of plasmid pcDNA3 containing the cDNA of HLA-B*4402. NA8-MEL melanoma cells (HLA-A1, A2, B12, B40, MAGE3-, 30000 cells/well) were co-transfected with 100 ng of plasmid pcDNA1/Amp-MAGE3 and either 100 ng of plasmid pcDNA3-HLA-B*4402 or 100 ng of plasmid pcDNA1/Amp-HLA-B*4403, using LIPOFECTAMINE (Gibco BRL). After 36 h of incubation at 37 °C, the COS-7 and NA8-MEL transfectants were tested for their ability to stimulate the production of TNF by CTL (Lehmann et al. 1995). In brief, 3000 CTL were added in 100 µl of Iscove's medium supplemented with 10% human serum and IL-2 (25 units/ml). After 20 h, the supernatant was collected and its TNF content determined by testing its cytotoxicity on WEHI-164c13 cells (Espevik and Nissen-Meyer 1986).

Chromium-release assay

Target cells shown in Figures 1 and 2 were labeled with ⁵¹chromium as described above, and washed. The labeled target cells (1000 cells/well) were incubated with the effector cells in the absence or presence of peptide MEVDPIGHLY (1 µg/ml) (Fig. 1). Alternatively, the labeled target cells (1000 cells/well) were first incubated in V-bottom micro-wells for 30 min at 20 °C with various concentrations of the peptide in serum-free medium X-VIVO 10 and washed in Iscove's medium containing 2% FCS, before addition of CTL (10000 cells/well) in medium containing 10% human serum (Fig. 2).

HLA-B44 subtyping

Part of exon 3 of the *HLA-B44* allele was amplified from genomic DNA with oligonucleotides BX3S1 (5'-GGGTCCAGGGTCTCATCA-3') and BX3R1 (5'-CCAGGTATCTGCGGAGCG-3'). The amplified 254 bp fragment of exon 3 was digested with *Rsa*I, *Pvu*II, and *Bsa*AI to distinguish between the subtypes B*4402, -03, -04, and -05.

Fig. 1 Lytic activities of CD8⁺ blood lymphocytes stimulated with peptide MAGE-3.B44. Two series, of six and five cultures, were set up with 2×10⁶ CD8 blood lymphocytes from normal donors LB816 (HLA-B*4402) and LB822 (HLA-B*4403), respectively. These lymphocytes were stimulated with autologous macrophages and dendritic cells incubated with peptide MAGE3.B44. The responder cells were restimulated every week by autologous cells incubated with peptide MAGE3.B44. On day 20, lytic activities were tested against EBV-transformed B cells expressing the same HLA-B44 subtype as the donor, namely LB33-EBV (HLA-B*4402) or LG2-EBV (HLA-B*4403). The indicated numbers of effector cells were incubated for 45 min with unlabeled K562 target cells (50000 cells/well) in order to inhibit lysis by NK-like effectors. ⁵¹chromium-labeled target cells (1000 cells/well), incubated with 1 µM peptide MAGE-3.B44 (●), or without it (○), were added and chromium release was measured after 4 h

Fig. 2 Recognition of peptide MAGE3.B44 by HLA-B*4402 or HLA-B*4403-restricted CTL clones. CTL clones (10000 cells/well) were added to the labeled lymphoblastoid B cells (1000 cells/well), which had been incubated for 30 min at 20 °C in serum-free medium with the indicated concentrations of peptide MAGE3.B44 and washed. Chromium release was measured after 4 h

Results and Discussion

HLA-B44 is expressed in 22%–24% of Caucasians (Imanishi et al. 1991; Lee 1990). Five *HLA-B44* alleles have been identified: *HLA-B*4402*, 3, 4, 5, and 6 (Fleischhauer et al. 1991; Petersdorf et al. 1994; Yao et al. 1994, 1995a, 1995b). The *HLA-B*4402* and *B*4403* alleles are expressed by 61% and 36% of *HLA-B44* Caucasians, respectively (Yao et al. 1995a). They differ only at position 156, located in the middle of the $\alpha 2$ helix and extending into the peptide binding site: it is occupied by Asp in *HLA-B*4402* and by Leu in *HLA-B*4403* (Fleischhauer et al. 1991). Bone marrow allograft rejection and graft versus host disease have been observed between donors and recipients differing with respect to these two subtypes of *HLA-B44*, suggesting that there might be differences in peptide presentation by the two subtypes (Fleischhauer et al. 1990; Keever et al. 1994). Therefore, in our search for a MAGE3 peptide recognized on *HLA-B44* we examined both the *HLA-B*4402* and *HLA-B*4403* subtypes.

Identification of MAGE3-encoded peptides binding to *HLA-B44*

Antigenic peptides recognized by T lymphocytes on *HLA-B44*, or eluted from *HLA-B44* molecules, often contain Glu in position 2 and Tyr or Phe in position 9 or 10 (Brichard et al. 1995; Buseyne et al. 1993; Coulie et al. 1995; DiBrino et al. 1995; Fleischhauer et al. 1994; Khanna et al. 1992; Kita et al. 1993). There are four peptide sequences with this binding motif in the *MAGE3* sequence (Table 1). These peptides were synthesized, and their ability to bind to *HLA-B44* molecules was tested in a competition assay. The MHC

Table 1 Binding to *HLA-B44* of peptides encoded by *MAGE3*. The ability of the peptides to compete with a standard peptide for binding to *HLA-B*4402* and *HLA-B*4403* molecules was measured with CTL clone 159/5, which recognizes peptide EEKLIVVAF on C1R-B*4402 or C1R-B*4403 cells. Specific lyses obtained with this peptide alone were 58% and 72% for C1R-B*4402 and C1R-B*4403, respectively. Control peptides included tyrosinase peptide SEIWRDIDF, which was shown to bind to both *HLA-B44* subtypes (Brichard et al. 1995), and EBNA3A peptide FLRGRAYGL, which binds to *HLA-B8* (Burrows et al. 1990)

Competitor peptides	Concentration (μ M) of peptide resulting in a 50% inhibition of the lysis of C1R-B44 cells sensitized with peptide EEKLIVVAF	
	C1R-B*4402	C1R-B*4403
MAGE3 peptides		
QEEGPSTF (90–98)	60	15
MEVDPIGHLY (167–176)	2	1
WEELSVLEVF (222–231)	80	7
EELSVLEVF (223–231)	20	5
Control peptides		
SEIWRDIDF (Tyrosinase)	1	<1
FLRGRAYGL (EBNA3A)	>100	>100

Fig. 3 Binding of peptide MEVDPIGHLY to *HLA-B44* molecules measured in a competition assay. The data were obtained in the experimental conditions described in Table 1. Competitor peptides included the MAGE3.B44 peptide MEVDPIGHLY (●), the tyrosinase.B44 peptide SEIWRDIDF (□) as positive control, and the EBNA3A peptide FLRGRAYGL (○) as negative control

class I-negative C1R cells were transfected with either the *HLA-B*4402* or the *HLA-B*4403* gene. The C1R-B44 cells were incubated with increasing concentrations of different *MAGE3* peptides and with a constant concentration of peptide EEKLIVVAF, which is recognized on *HLA-B44* by the anti-melanoma cytolytic T-cell (CTL) clone LB33-CTL-159/5 (Coulie et al. 1995). All four *MAGE3* peptides inhibited lysis of C1R-B44 cells, indicating that they bound to *HLA-B44* molecules (Table 1). Peptide MEVDPIGHLY was the best binder on both *HLA-B*4402* and *B*4403* molecules. It competed nearly as efficiently as a tyrosinase peptide, which is recognized by CTL clones on *HLA-B*4402* or *HLA-B*4403* melanoma cells (Brichard et al. 1995; Fig. 3). Peptides homologous to peptide MEVDPIGHLY, derived from the *MAGE1*, 2, 4, 6, and 12 genes, and containing the *HLA-B44* binding motif, were also able to bind to *HLA-B44* (data not shown).

The sequence of peptide MEVDPIGHLY includes that of the *MAGE3*.A1 peptide, EVDPIGHLY, which we knew to be processed in cells expressing *MAGE3*, since it is recognized by *HLA-A1*-restricted CTL on *MAGE3*⁺ melanomas (Gaugler et al. 1994). Moreover, peptide MEVDPIGHLY showed significant binding in vitro to purified soluble TAP1/TAP2 heterodimers (Fleischhauer et al. manuscript in preparation). Therefore anticipating that peptide MEVDPIGHLY may also be presented by cells expressing *MAGE3*, we focused on this peptide, referred to below as *MAGE3*.B44.

Analysis of alanine-substituted variant peptides indicated that the side chain of the carboxyterminal tyrosine of nonamer MAGE3.A1 and decamer MAGE3.B44 was critical in anchoring these peptides into *HLA-A1* and *HLA-B44* (Gaugler et al. 1994, and data not shown). But the other anchor residue appeared to be different for each

Fig. 4 Recognition of transfected cells expressing HLA-B44 and MAGE3 by MAGE3.B44-specific CTL clones. The MAGE3 and HLA-B*4403 cDNAs were cloned into expression plasmid pcDNA1/Amp, and the HLA-B*4402 cDNA was cloned into expression plasmid pcDNA3. COS-7 (10000/well) and NA8-MEL (30000/well) cells were transfected using DEAE-dextran and liposomes, respectively. After 36 h, CTL (3000/well) were added to the transfectants and after a further 20 h the concentration of TNF released in the medium was measured with the TNF-sensitive WEHI-164c13 cells

peptide: Asp in position 3 was critical for binding of the nonamer to HLA-A1, and Glu in position 2 for the decamer's binding to HLA-B44. The nonamer did not bind detectably to HLA-B*4402 molecules, but it bound to HLA-B*4403 about ten times less efficiently than the decamer. In addition to binding to HLA-A1 and B44, the nonamer bound also to HLA-A29 molecules, through residues in positions 2, 5, 8, and 9 (I. F. Luescher, manuscript in preparation). This type of peptide binding to different HLA molecules through different residues differs from the previously described "supermotifs" which allow peptide binding to different HLA molecules through the same main anchor residues (del Guercio et al. 1995; Sidney et al. 1995).

Induction of CTL recognizing peptide MAGE3.B44

Adherent PBMC were isolated from healthy donor LB816, whose HLA-type was HLA-B*4402. They were cultured for 7 days in the presence of GM-CSF and IL-4 in order to increase the proportion of dendritic cells (Romani et al. 1994; Sallusto and Lanzavecchia 1994). These cells were incubated with peptide MAGE3.B44 (50 μ M), irradiated, and used as stimulator cells in six cultures of 2×10^6 CD8⁺ blood T lymphocytes of the donor in medium containing IL-6 and IL-12. On days 7 and 14, the responder cells were restimulated with irradiated autologous PBMC incubated with the peptide in medium containing IL-2 and IL-7, and on day 20 the lytic activity was assessed on HLA-B*4402 lymphoblastoid B cells sensitized with peptide MAGE3.B44 (Fig. 1A). Only one of the six populations of responder cells preferentially lysed the peptide-pulsed target cells. The same procedure was used to derive MAGE3.B44-specific lytic effectors from PBMC of the HLA-B*4403 healthy donor LB822. One of five populations of responder cells appeared to contain anti-peptide CTL (Fig. 1B). These results suggested that under these conditions the frequency of precursors of MAGE3.B44-specific CTL was of the order of $1/10^7$ CD8⁺ lymphocytes.

Fig. 5 Lytic activity of MAGE3.B44-specific CTL clones on HLA-B44 tumor cell lines expressing MAGE-3. CTL clone 340/1, derived from the HLA-B*4402 donor, was tested against HLA-B*4402 tumor cells, and CTL clone 346/8, derived from the HLA-B*4403 donor, was tested against HLA-B*4403 tumor cells. Target cells included MAGE3⁺ melanoma cell lines, MZ2-MEL.61.2 cells which have lost expression of gene-MAGE-3 (○), and one MAGE3⁺ bladder carcinoma cell line. All target cells, except MZ2-MEL.43, MZ2-MEL.61.2 and LG2-MEL, were incubated over 48 h with IFN γ (50 units/ml) prior to the lysis assay. Expression of HLA-B*4402, HLA-B*4403, and MAGE3 by the various target cells was tested by RT-PCR. Chromium release was measured after 5 h

The lymphocytes of the two positive cultures were cloned by limiting dilution. CTL clone LB816-CTL-340/1 lysed HLA-B*4402 EBV-transformed B cells incubated with peptide MAGE3.B44. CTL clone LB822-CTL-346/8 recognized the MAGE3.B44 peptide presented by HLA-B*4403 cells (Fig. 2). Half-maximal lysis was obtained at a peptide concentration of approximately 40 nM for CTL 340/1 and 100 nM for CTL 346/8.

The MAGE6-encoded peptide, which is homologous to the MAGE3.B44 peptide and differs from it only at position 9 (L $\rightarrow$ V), was not recognized by the MAGE3.B44-specific CTL clones, although this peptide bound efficiently to both HLA-B44 subtypes. The leucine residue in position 9 of peptide MAGE3.B44 is therefore part of the epitope recognized by the receptors of the two CTL clones.

Recognition by CTL of HLA-B44 cells expressing the MAGE3 gene

To determine whether the MAGE3.B44 peptide was naturally presented by HLA-B44 on cells that expressed MAGE3, COS-7 cells were co-transfected with MAGE3 and HLA-B*4402 or HLA-B*4403 cDNA clones. These

cDNAs had been inserted into expression vectors carrying the SV40 origin of replication, which multiply in large numbers in COS-7 cells, because these cells express the SV40 T product (Gluzman 1981). The cells transfected with the two constructs stimulated TNF release by CTL clones 340/1 and 346/8, whereas those transfected with *HLA-B44* or *MAGE3* alone did not (Fig. 4). Similar results were obtained when the melanoma cells NA8-MEL, which express neither *HLA-B44* nor *MAGE3*, were cotransfected with *HLA-B44* and *MAGE3* cDNA clones. Recognition of the MAGE3/HLA-B44 antigen therefore does not require the very high gene copy number present in COS-7 cells.

The two MAGE3.B44-specific CTL clones also efficiently lysed several HLA-B44 tumor cell lines which expressed the *MAGE3* gene (Fig. 5). Tumor cell lines that did not express *MAGE3* or *HLA-B44* were not lysed by the CTL clones.

The MAGE3.B44 peptide may prove useful for specific immunization of HLA-B44 cancer patients. In several types of tumors, *MAGE3* is the most frequently expressed *MAGE* gene: 76% of metastatic melanomas (Brasseur et al. 1995), 48% of head and neck tumors, 35% of bladder carcinomas (Patard et al. 1995), 31% of lung carcinomas (Weynants et al. 1994), 24% of sarcomas and 12% of breast cancers (Russo et al. 1995). The identification of a MAGE3/HLA-B44 antigen increases the proportion of cancer patients eligible for immunization against MAGE-encoded antigens. With regard to metastatic melanoma, approximately 17% of caucasian patients should express this antigen, since 24% of individuals are *HLA-B44*, and 76% of metastatic melanomas express the *MAGE3* gene. In addition, 37% of patients express antigen MAGE-3/HLA-A2 and 20% express antigen MAGE3/HLA-A1. Around 53% of metastatic melanoma patients express at least one of the MAGE3-encoded antigens.

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