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Immunology

Induction of anti-tumor cytotoxic T lymphocytes in normal humans using primary cultures and synthetic peptide epitopes

(major histocompatibility complex antigen-binding peptides/tumor-specific cytotoxic T cells/MAGE antigens/melanoma immunity)

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ABSTRACT **Cytotoxic T lymphocytes (CTLs) recognize peptide antigens associated with cell surface major histocompatibility complex (MHC) molecules. The identification of tumor cell-derived peptides capable of eliciting anti-tumor CTL responses would enable the design of antigen-specific immunotherapies. Our strategy to identify such potentially therapeutic peptides relies on selecting high-affinity MHC binders from known tumor-associated antigens. These peptides are subsequently tested for their ability to induce CTLs capable of killing tumor cells. With this strategy, we have identified a nine-residue epitope, derived from the product of the tumor-associated gene *MAGE-3*, which has the capacity to induce *in vitro* CTLs that kill melanoma and other tumor cell lines. These results show the primary *in vitro* induction of tumor-specific human CTLs and illustrate the feasibility of *ex vivo* antigen-specific approaches to the immunological therapy of cancer.**

With the appropriate manipulation, the immune system has the ability to mount responses that can destroy tumor cells (1–4). Among the various elements that constitute the immune system, cytotoxic T lymphocytes (CTLs) have the potential of being the most effective in mediating the rejection of established tumors (1–4). The reason for this potential is that while antibodies with anti-tumor activity must recognize and bind integral cell surface molecules, CTLs can react against antigenic determinants produced from any protein synthesized within the cell (5, 6).

The anti-tumor activity displayed by tumor-specific CTLs is the result of a series of complex molecular events. More specifically, subsequent to the normal cellular processing of proteins in the cytoplasm of the tumor cells, small peptides are transported to the endoplasmic reticulum, where they bind to newly synthesized major histocompatibility complex (MHC) class I molecules. MHC/peptide complexes are then exported to the surface of the tumor cell, where they can be recognized by antigen-specific class I-restricted CTLs (5–10). In addition to lysing the tumor cell, the CTLs may also secrete lymphokines such as tumor necrosis factor and γ-interferon (γ-IFN), which also contribute to the overall anti-tumor effect (1–3).

Peptide binding to MHC class I molecules is contingent on the allelic type of the MHC molecule and the amino acid sequence of the peptide (11–15). MHC class I-binding peptides, which are in general 9–10 residues long, usually contain within their sequence two conserved ("anchor") residues that interact with corresponding binding pockets in the MHC molecule. Specific combinations of anchor residues (usually referred to as "MHC motifs") required for binding by several allelic forms of human MHC (HLA) antigens have been defined (11, 14, 15). Definition of specific MHC motifs allows us to predict from the amino acid sequence of an individual protein, such as viral products or tumor-associated molecules, those peptides that have the potential of being antigenic for CTLs.

For the immune system to mount an effective CTL response against tumor-derived peptides, these peptides must not only be capable of binding to the particular class I MHC molecules expressed by the tumor cell but must also be recognized by the pool of available T-cell specificities. Thus, mutations of genes encoding cellular proteins (such as oncogenes) and/or the overexpression of genes coding for regulatory proteins (such as, tumor suppressor proteins) could result in the production of potential tumor-specific immunogenic peptides. Boon and collaborators (16) have described a family of ≈12 genes of yet undefined function (called *MAGE*, for melanoma antigen), some of which are selectively expressed in a variety of human malignant cells (particularly in melanomas), but not in most normal tissues, with the exception of the testis (16). For example, the *MAGE-1* gene is expressed in ≈40% of melanomas and in some other tumors. Indeed, a recent report described the isolation from a melanoma patient of CTLs specific for a 9-residue peptide (Glu-Ala-Asp-Pro-Thr-Gly-His-Ser-Tyr), derived from the processing of the MAGE-1 protein (occupying positions 161–169; ref. 16). Further, these CTLs recognized the MAGE-1-derived peptide in the context of the HLA-A1 molecule (17).

In contrast with the somewhat limited frequency of expression of *MAGE-1*, the *MAGE-2* and *MAGE-3* genes are expressed in 80–90% of the melanoma lines examined and also in the other tumor types such as breast, colon, lung, and thyroid cancers (16, 18). Thus, it would be attractive to identify peptides derived from the *MAGE-2* or *MAGE-3* gene products which could serve as CTL antigens.

Here we describe a strategy for the identification of CTL-immunogenic/antigenic peptides which were derived from the sequence of the *MAGE-2* and *-3* genes. We have chosen the HLA-A1 system for this study because CTLs specific for a MAGE-1-derived peptide have already been described (17). Our strategy consisted of first selecting and synthesizing peptides bearing MHC-binding motifs for HLA-A1 from the known *MAGE-2* and *-3* gene sequences (16). These synthetic peptides were then tested for their capacity to bind to purified HLA-A1 molecules, and finally, the high-affinity MHC-binding peptides were studied for their ability to elicit a primary CTL response from lymphocytes from normal donor volunteers. Using this strategy we have identified one peptide derived from the MAGE-3 sequence that elicited CTLs capable of killing MAGE-3$^+$ tumor cells. Interestingly, this peptide from MAGE-3 corresponds to the same protein

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Abbreviations: CTL, cytotoxic T lymphocyte; MHC, major histocompatibility complex; IL, interleukin; IFN, interferon; APC, antigen-presenting cell; PBMC, peripheral blood mononuclear cell.

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region which had been shown to be antigenic for CTLs specific for MAGE-1 (17).

The approach described here should be applicable to any MHC class I-restricted CTL system where the sequence of the antigen and MHC anchor peptide motifs are known and the corresponding MHC peptide-binding assays have been developed.

MATERIALS AND METHODS

Synthetic Peptides. Peptides used for MHC binding assays were purchased from Chiron/Mimotopes. For cellular assays, synthetic peptides were prepared on an Applied Biosystems machine. Briefly, after removal of the α-amino-protecting *tert*-butyloxycarbonyl group, the phenylacetamidomethyl resin peptide was coupled with a 4-fold excess of preformed symmetrical anhydride (hydroxybenzyltriazole esters for arginine, histidine, asparagine, and glutamine) for 1 hr in dimethylformamide. For arginine, histidine, asparagine, glutamine, and histidine residues, the coupling step was repeated to obtain a high efficiency of coupling. Peptides were cleaved by treatment with hydrogen fluoride in the presence of the appropriate scavengers. Synthetic peptides were purified by reverse-phase high-pressure liquid chromatography. The purity of the peptides, which was routinely >95%, was determined by amino acid sequence and/or composition analysis.

Purification of HLA-A1 Molecules. For the peptide–MHC binding assays the HLA-A1 molecules were purified by a procedure similar to the one described recently (13) except that Steinlin cells (*HLA-A1*-homozygous) were used as a source of MHC molecules. Briefly, detergent extracts of Steinlin cells were first depleted of HLA-B and -C antigens by repeated passage over a column prepared with monoclonal antibody B1.23.2, which is specific for these molecules (19). HLA-A1 molecules were subsequently purified by affinity chromatography using an immunoadsorbent prepared with antibody W6/32, which recognizes all human MHC class I molecules associated to β_2-microglobulin (20). The adsorbed HLA-A1 molecules were eluted from the column with base (50 mM diethylamine) containing 1% octyl β-D-glucoside. The eluted HLA-A1 molecules were neutralized and dialyzed against phosphate-buffered saline containing 1% octyl glucoside and concentrated by ultrafiltration. Purity of HLA-A1 molecules was determined to be >90% by SDS/PAGE.

MHC Binding Assay. Peptide binding to class I MHC molecules was measured as described (13). Briefly, the assay for peptide binding to purified MHC class I molecules is based on the inhibition of binding of the radiolabeled standard peptide ^{125}I-labeled Tyr-Leu-Glu-Pro-Ala-Ile-Ala-Lys-Tyr to detergent-solubilized MHC molecules. The sequence of this standard HLA-A1-binding peptide was selected based on a "consensus" sequence of HLA-A1-binding peptides (A.S., unpublished data). The standard peptide was radioiodinated with ^{125}I (ICN) by the chloramine-T method. HLA-A1 concentrations yielding ≈15% of bound peptide (approximately in the 10 nM range) were used in the inhibition assays. Various doses of the test peptides (10 μM to 1 nM) were incubated together with 5 nM radiolabeled standard peptide and HLA-A1 molecules for 2 days at room temperature in the presence of a mixture of protease inhibitors and 1 μM β_2-microglobulin (Scripps Laboratories, San Diego). At the end of the incubation period, the percent MHC-bound radioactivity was determined by gel filtration.

Cell Lines. The Steinlin cell line (HLA-A1/1, -B8/8) was obtained from the American Society for Histocompatibility and Immunogenetics Cell Repository (Brigham and Women's Hospital, Boston). The HLA-typed melanoma cell lines were a generous gift from S. Rosenberg, National Cancer Institute, Bethesda, MD. The breast carcinoma lines HBL-100 and BT-20 and the prostate cancer line PC3 were obtained from the American Type Culture Collection. The .221(A1) and .221(A2.1) cells were produced by transferring the *HLA-A1* and *-A2.1* genes into the HLA-A, -B, -C-null mutant human B-lymphoblastoid cell line .221 (21). The HLA genes were first removed from their pHeBo vectors and cloned into the RSV.5neo vector (22) to allow the derivation of genetically stable transferrent cells. After transfer of the plasmids into .221 cells, the .221(A1) and .221(A2.1) transferrent clones were isolated by selection in medium containing the neomycin analog G418 (Sigma) at 400 μg/ml. Flow cytometry of anti-class I MHC antibody binding showed that .221(A1) and .221(A2.1) cells expressed 50–100% as much cell surface HLA-A as did normal lymphoblastoid cells expressing single copies of the same HLA alleles in their native chromosomal locations. All tumor lines were maintained in culture using RPMI-1640 medium supplemented with antibiotics and 10% (vol/vol) fetal bovine serum [and G418 at 400 μg/ml for the .221(A1) and .221(A2.1) cell lines]. Some cells were treated with γ-IFN (100 units/ml; Genzyme) for 72 hr at 37°C before their susceptibility to lysis by the CTLs was tested. In all cases, the γ-IFN-treated cells increased the level of MHC class I expression by a factor of 2–3.

Primary CTL Induction Using Synthetic Peptide. CTLs were elicited using synthetic peptides in normal blood donor volunteers (unpublished work by E.C. and P.A.W.). Briefly, peripheral blood mononuclear cells (PBMCs) from a normal volunteer (HLA-A1/24, -B8/38. -Cw7) were purified by centrifugation in Ficoll-Paque (Pharmacia). The Institutional Review Board on Human Subjects (Cytel, San Diego) approved this research, and informed consent for blood donations was obtained from all volunteers. Nontransformed lymphoblasts were used as antigen-presenting cells (APCs) and were prepared by incubation of PBMCs (2×10^6 per ml) in 24-well tissue culture plates for 4–6 days with *Staphylococcus aureus* Cowan-I (Pansorbin, Calbiochem) at 0.005% (vol/vol) and rabbit anti-human IgM antibody at 20 μg/ml coupled onto a solid phase (Immunobeads, Bio-Rad) in the presence of recombinant interleukin 4 (IL-4, 20 ng/ml; Sandoz Pharmaceutical). Culture medium for the preparation of these APCs consisted of RPMI 1640 (GIBCO) supplemented with 10% fetal bovine serum (GIBCO). Pansorbin-induced lymphoblasts (10^6 per ml) were incubated overnight at 26°C in a 5% CO_2 incubator and were loaded with peptide at 50 μg/ml in the presence of β_2-microglobulin at 3 μg/ml for 4 hr at 20°C in phosphate-buffered saline. These APCs were γ-irradiated with 6000 rads (1 rad = 0.01 Gy), resuspended at 10^6 cells per ml, and mixed at a ratio of 1:3 with autologous $CD4^+$ cell-depleted PBMCs (responder cells). Depletion of $CD4^+$ cells was carried out using anti-CD4 antibody-coated magnetic beads (Dynabeads, Oslo). The cultures were maintained in RPMI 1640 medium supplemented with 5% heat-inactivated AB human serum and recombinant IL-7 (10 ng/ml, Genzyme), for 12 days at 37°C in a 5% CO_2 incubator. Recombinant IL-2 (Sandoz Pharmaceutical) was added to the cultures at 10 units/ml on days 12–14. The responder cells were restimulated every 7–10 days in 24-well plates, at 10^6 cells per well, with autologous irradiated adherent cells, which were incubated with peptide. To prepare the adherent APCs, 4×10^6 irradiated (3500 rads) autologous PBMCs in 0.5 ml of medium were added to each well of a 24-well tissue culture plate. After 2 hr at 37°C, the nonadherent cells were gently removed, and the adherent cells were incubated for 2 hr with peptide (10 μg/ml) and β_2-microglobulin (3 μg/ml) in a final volume of 0.5 ml of medium per well. The excess peptide was removed, and 1 ml of the responder cell population was added to each well. The cultures were fed every 3–5 days with fresh medium containing recombinant IL-2. Cytotoxicity was first tested after two rounds of antigenic stimulation (day 19). Cytolytic activity of the T-cell line

increased after every restimulation. The MAGE-3-reactive CTL line has been maintained in tissue culture for >4 months.

CTL Cytotoxicity Assays. Adherent target cells (melanomas and prostate and breast tumor cells) were detached from tissue culture flasks with 1 mM EDTA in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline. All cells were labeled with 100 μCi (3.7 MBq) of ^{51}Cr (ICN) per 10^6 cells for 1 hr at 37°C. Peptide-pulsed targets were prepared by incubating the cells (10^6) with synthetic peptide (10 μg/ml) either for 1 hr (during the ^{51}Cr labeling) or overnight at 37°C. Target cells were washed by centrifugation and mixed with various numbers of effectors in a final volume of 0.2 ml of RPMI 1640 containing 10% fetal bovine serum (GIBCO) in round-bottom microtiter plates. After 4–6 hr of incubation, the plates were centrifuged (5 min at 400 × *g*), 0.1 ml of the supernatant was removed from each well, and the radioactivity was determined in a γ counter.

Percent specific cytotoxicity was determined by calculating the percent specific ^{51}Cr release: [(cpm of the test sample − cpm of the spontaneous ^{51}Cr release)/(cpm of the maximal ^{51}Cr release − cpm of the spontaneous ^{51}Cr release)] × 100. The spontaneous ^{51}Cr release was determined by incubating the targets alone, in the absence of effectors, and the maximal ^{51}Cr release was obtained by incubating the targets with 0.1% Triton X-100 (Sigma). All determinations were done in duplicates, and the standard errors of the means were always <10% of the value of the mean.

RESULTS

Identification of Peptides from MAGE-2 and -3 That Bind to HLA-A1. Initially, the known sequences of MAGE-2 and -3 (16) were screened for peptides containing the anchor motif for HLA-A1 (Thr/Ser/Met in position 2, or Asp/Glu/Ala/Ser/Thr in position 3, and Tyr in the carboxyl-terminal end; R. Kubo, personal communication). Ten peptides (7 from MAGE-3 and 3 from MAGE-2) of either 9 or 10 residues were found to contain the binding motif for HLA-A1. These peptides were synthesized and tested for binding to purified HLA-A1 molecules. The results from the binding assay showed that the relative binding affinities of these peptides for HLA-A1 ranged over 4 orders of magnitude (Table 1). It was found that 2 peptides, both from MAGE-3, bound to HLA-A1 with high affinity (<50 nM required to achieve 50% inhibition), 3 peptides were intermediate HLA-A1 binders (50–500 nM required for 50% inhibition), and the remaining 5 peptides bound HLA-A1 only weakly (500 nM to 50 μM to achieve 50% inhibition). In addition, 7 peptides (3 nonamers and 4 decamers) that do not have HLA-A1-binding motifs, from the sequences of MAGE-2 and -3, did not bind to this MHC molecule (Table 1).

Interestingly, the highest binding peptide to HLA-A1 was the MAGE-3 homolog, EVDPIGHLY (one-letter code), of the previously described MAGE-1 CTL epitope, EADPTGHSY (17). Fig. 1 shows a comparison of the binding capacity of the 3 peptides derived from homologous regions of the different products derived from *MAGE-1*, *-2*, and *-3* genes. The data indicate that both the MAGE-1-derived peptide, EADPTGHSY, and the peptide derived from MAGE-3, EVDPIGHLY, bound with high affinity to HLA-A1. On the other hand, the peptide derived from the homologous region of MAGE-2, EVVPISHLY, which does not contain one of the crucial anchor residues (D in position 3) bound very poorly (10,000-fold less than the others) to purified HLA-A1.

Induction of Primary CTL Responses to MAGE-2- and -3-Derived Peptides. Previous observations have revealed that >95% of the peptides corresponding to known CTL epitopes belong to the high or intermediate MHC-binding groups (A.S., unpublished work). Following this rationale, we proceeded to study whether the six highest MHC-binding peptides from MAGE-2 and -3 could be effective in raising HLA-A1-restricted CTLs. Using an *in vitro* primary CTL induction protocol recently developed (P.A.W., unpublished work), we stimulated PBMCs from at least two normal HLA-A1 volunteers with the synthetic peptides. Out of the six peptides studied, only one, the highest MHC binder (EVDPIGHLY), from MAGE-3, was able to elicit CTLs in one of the blood donors. After two rounds of stimulation in culture with autologous APCs pulsed with peptide EVDPIGHLY, significant cytotoxic activity toward peptide-sensitized, HLA-A1-bearing target cells was observed (Fig. 2*A*). More significant was the observation that two MAGE-3-expressing HLA-A1 melanoma cell lines (397-mel and 938-mel; ref. 18) were also killed by these CTLs (Fig. 2*A*). No anti-peptide or anti-tumor reactivities were detected in the case of the remaining five potential CTL epitopes, despite their screening in at least four independent HLA-A1$^+$ blood donors.

Antigen Specificity and MHC Restriction Analysis. The CTL response appeared to be specific and HLA-A1-restricted, since HLA-A1 melanoma cells negative for MAGE-3 (mel-888), and melanoma cells that expressed MAGE-3 but were of a different HLA-A allelic type (mel-526; HLA-A2/A3; ref.

Table 1. HLA-A1 binding of motif containing nonapeptides and decapeptides derived from the sequences of MAGE-2 and MAGE-3

Source	Position*	Sequence	HLA-A1 motif	Size	Binding (IC_{50},† nM)
MAGE-3	161	EVDPIGHLY	+	9	4.6
MAGE-3	61	ASSLPTTMNY	+	10	9.6
MAGE-2	61	ASSFSTTINY	+	10	147.1
MAGE-3	62	SSLPTTMNY	+	9	454.6
MAGE-3	130	GSVVGNWQY	+	9	500.0
MAGE-2	62	SSFSTTINY	+	9	581.4
MAGE-3	171	FATCLGLSY	+	9	657.9
MAGE-2	171	LVTCLGLSY	+	9	2976.2
MAGE-3	129	LGSVVGNWQY	+	10	12,500
MAGE-3	170	IFATCLGLSY	+	10	12,500
MAGE-2	105	KMVELVHFLV	−	9	>36,000
MAGE-2	105	KMVELVHFLL	−	10	>36,000
MAGE-3	153	LVFGIELMEV	−	10	>36,000
MAGE-3	105	KVAELVHFL	−	9	>36,000
MAGE-3	182	LLGDNQIMPK	−	10	>36,000
MAGE-3	115	LYIFATCLGL	−	10	>36,000
MAGE-3	16	NYPLWSQSY	−	9	>36,000

*Refers to residue number of the first position of the peptide in relation to the sequence of the entire gene product.
†Concentration of the peptide necessary to inhibit 50% binding of the radiolabeled test peptide to purified HLA-A1 molecules.

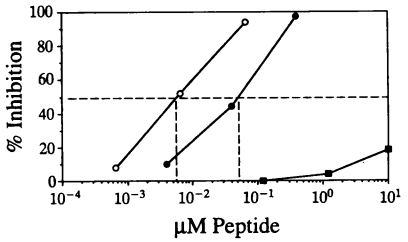

FIG. 1. HLA-A1 binding of synthetic peptides from MAGE-1, -2, and -3. The MAGE peptides were tested in a dose titration for the inhibition of the binding of the ^{125}I-labeled standard peptide YLEPAIAKY to purified HLA-A1 molecules. ●, MAGE-1 peptide EADPTGHSY; ■, MAGE-2 peptide EVVPISHLY; ○, MAGE-3 peptide EVDPIGHLY. Dotted lines are used to calculate the 50% inhibitory dose for each peptide.

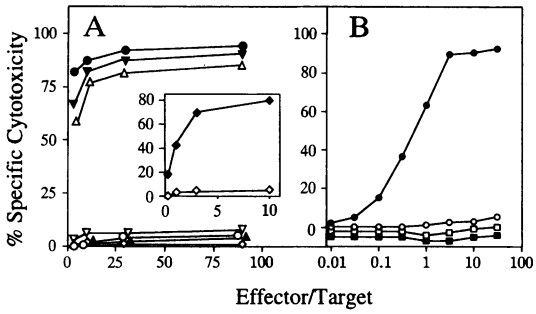

FIG. 2. Antigen specificity and MHC restriction analysis of MAGE-3-reactive CTLs. (*A*) Cytotoxic responses using peptide-loaded target cells and melanoma tumors. ●, Steinlin (*HLA*-homozygous, Epstein–Barr virus-transformed lymphoblastoid cell line, HLA-A1/1, -B8/8) pulsed with MAGE-3 peptide EVDPIGHLY; ▲, Steinlin cells pulsed with MAGE-1 peptide EADPTGHSY; ○, Steinlin cells with no peptide; ▼, mel-397 (HLA-A1/25, MAGE-3$^+$); △, mel-938 (HLA-A1/24, -B7/8, MAGE-3$^+$); ◇, mel-888 (HLA-A1/24, -B22/52, -Cw1/w7 MAGE-3$^-$); ◆, mel-888 pulsed with MAGE-3 peptide EVDPIGHLY; ▽, mel-526 (HLA-A2/3, -B50/62, -Cw3, MAGE-3$^+$). (*B*) Cytotoxic responses using peptide-loaded HLA-mutant cells expressing single MHC class I molecules (21). ●, .221(A1) pulsed with MAGE-3 peptide EVDPIGHLY; ○, .221(A1) without peptide; ■, .221(A2.1) pulsed with MAGE-3 peptide EVDPIGHLY; □, .221(A2.1) without peptide.

18) were not lysed by the CTL line. Addition of the peptide EVDPIGHLY to the MAGE-3-negative, HLA-A1-positive mel-888 cell line rendered the cells susceptible to lysis by the CTLs (Fig. 2*A Inset*), indicating that this tumor expresses functional HLA-A1 molecules and can function as a CTL target. Further, the CTL line did not crossreact with the product of the *MAGE-1* gene, since it failed to kill HLA-A1 targets loaded with peptide EADPTGHSY derived from the MAGE-1 sequence (Fig. 2*A*). Thus, it appears that one or more of the three amino acids that are different between EVDPIGHLY and EADPTGHSY (at positions 2, 5, and 8) may function as T-cell receptor contact residues. Under the conditions used to propagate the CTL line, no significant natural killer or lymphokine-activated killer activity has been observed, since these cells did not kill the K-562 leukemia cell line (data not shown).

Supporting the conclusion that the restriction element for the recognition of peptide EVDPIGHLY was HLA-A1 were the results obtained using targets derived from mutant MHC class I-negative cell lines which were transfected with and expressed individual HLA-A (A1 or A2.1) genes (19). Only peptide-pulsed HLA-A1-transfected targets were killed by the CTLs, but not those expressing HLA-A2.1 molecules (Fig. 2*B*).

The MAGE-3 specific CTL line recognized the peptide EVDPIGHLY in a dose-related fashion (Fig. 3). These CTLs expressed the CD3 and CD8 T-cell surface makers typically found on most MHC class I-restricted CTLs. Furthermore, antibodies to the CD8 molecule, or to human MHC class I molecules (w6/32), were found to inhibit significantly (>80%) the cytotoxic function of the T-cell line (data not shown).

Cytolytic Activity on Non-Melanoma Tumors. As mentioned above, other tumors besides melanomas can also express *MAGE* genes—in particular, *MAGE-2* and *-3* (16, 18). We therefore tested the ability of the MAGE-3-specific CTL line to kill HLA-A1-expressing breast and prostate carcinoma cell lines. The results in Fig. 4 show that one of the two breast cancer cell lines (HBL-100), reported to express *MAGE-3* (18), was highly susceptible to lysis by the CTLs. The level of lysis improved significantly if these cells were previously incubated with γ-IFN (Fig. 4), which increased 2- to 3-fold the expression of MHC class I molecules on the cell

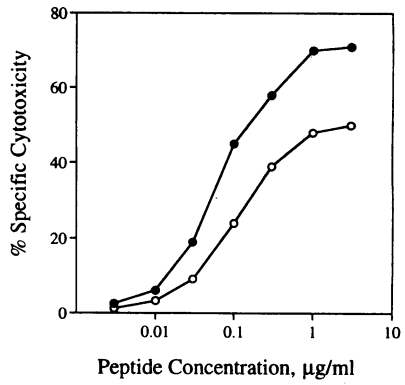

FIG. 3. Peptide dose–response of MAGE-3-specific CTLs. Peptide EVDPIGHLY was tested at various concentrations for its ability to sensitize Steinlin target cells. Synthetic peptide and target cells were first mixed in 96-well culture plates and incubated for 15 min at room temperature before the CTLs were added to the assay. Cytotoxicity was determined using two effector/target cell ratios: 5:1 (●) and 1:1 (○).

surface. The other breast cancer cell line (BT-20), also reported to express *MAGE-3* (18), and the prostate cancer line (PC3) were also killed, but to a lesser extent, and only when previously incubated with γ-IFN.

DISCUSSION

We have shown the feasibility of a systematic approach to rapidly identify CTL epitopes in proteins of known sequences. Potential antigenic peptides are first selected from the sequence of the putative antigenic protein (e.g., tumor-associated antigen, viral protein), for the presence of appropriate MHC anchor residues and then are selected for their capacity to bind with a good affinity to the relevant MHC molecule. Following this approach, we have identified a nonapeptide derived from the sequence of MAGE-3 that was able to elicit an *in vitro* primary CTL response in humans. The use of peptide fragments to elicit CTLs by primary *in vitro* stimulation using spleen cells (23) or dendritic cells (24) as APCs in the mouse system was reported several years ago. However, in these examples, either a nondefined mixture of peptide fragments from a cleaved protein (23) or a synthetic peptide from a known T-cell epitope (23, 24) was used to

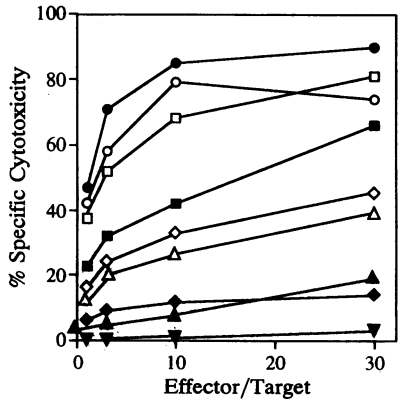

FIG. 4. Cytotoxic activity of MAGE-3-specific CTLs toward various tumors. The MAGE-3 specific CTLs were tested for their ability to kill breast and prostate HLA-A1$^+$ tumor lines previously treated or not treated with γ-IFN. Cytotoxic responses were measured against mel-397 (MAGE-3$^+$) (●), mel-397 plus γ-IFN (○), HBL-100 (HLA-A1/10, -B7/8 MAGE-3$^+$) (■), HBL-100 plus γ-IFN (□), BT-20 (HLA-A1, -B16.MAGE-3$^+$) (▲), BT-20 plus γ-IFN (△), PC3 (HLA-A1/9) (◆), PC3 plus γ-IFN (◇), and mel-888 plus γ-IFN (MAGE-3$^-$) (▼).

generate the CTLs. The present report constitutes an example of *in vitro* primary CTL induction in the human system, using a synthetic peptide identified by its ability to bind to MHC, resulting in effector cells that killed tumor cells. In addition to the CTL induction example presented here, CTLs of similar specificity and tumor reactivity were generated by using the same peptide and cells from a different HLA-A1 normal volunteer, but with the use of another type of APCs, PBMCs which were enriched for dendritic cells (V.T. unpublished work).

Although 10 peptides from MAGE-2 and -3 carried the appropriate HLA-A1 MHC-binding motif, only 6 of them were able to bind strongly to HLA-A1 molecules, and only 1 out of these 6 peptides, the highest MHC binder, was shown to date to induce primary CTL responses in our hands. These results point to the importance of the combined use of motif analysis and MHC binding assays in the selection and identification of CTL-immunogenic peptides. With the help of the MHC motifs and binding assays, the number of peptides to be screened for CTL induction can be significantly reduced. An example of a similar approach was recently described for peptides derived from the sequence of the p53 tumor-suppressor gene which contain HLA-A2.1-binding motifs (25). However, the main difference between that particular report (25) and the present work is that the p53 peptide-specific CTLs were not tested for their lytic activity using tumor target cells which would naturally process and present p53-derived CTL epitopes. Thus, it remains unknown whether the p53 sequences identified as potential CTL epitopes (25) are found associated with HLA-A2.1 molecules on the surface of tumor cells.

CTLs induced with the MAGE-3 peptide were found to efficiently kill several MAGE-3-positive tumor cell lines (Figs. 2*A* and 4), indicating that this peptide is expressed in association with HLA-A1 on the surface of tumor cells as a result of the processing of the product of the *MAGE-3* gene. The marked differences between the degree of CTL lysis observed between the melanoma cell lines and the other tumors (breast and prostate) could be due to variations in the level of surface HLA-A1 molecules and/or differences in the amounts of *MAGE-3* product that are processed and presented. The increased susceptibility of lysis by pretreatment with γ-IFN observed with some tumor cells (Fig. 4) is indeed compatible with the first possibility.

It is interesting that the HLA-A1-restricted MAGE-3 CTL epitope identified here and the HLA-A1-restricted MAGE-1 epitope previously identified by following a different and independent approach (16, 17) are derived from exactly the same protein region (residues 161–169). This result could be explained by the fact that the two homologous peptides, EADPTGHSY and EVDPIGHLY, derived from this region of MAGE-1 and -3 are the highest-affinity binders to HLA-A1 for each protein (Table 1; data not shown for MAGE-1). It is also possible that the processing of both *MAGE-1* and *-3* gene products results in the preferential production of HLA-A1-binding peptides that are derived from this region of the tumor-associated antigen.

Finally, our results suggest the possible therapeutic use of synthetic peptides to elicit CTL responses to melanoma, breast, colon, prostate, or other tumors which may express the *MAGE-3* or *MAGE-2* gene. The use of peptides derived from these gene products would be preferable to peptides from the *MAGE-1* gene, because of their higher frequency of expression in all of these tumor types (16, 18). The same technique could also be used for sequences of other tumor-associated proteins such as products of mutated oncogenes and tumor-suppressor genes. This approach could be used therapeutically either in the form of a peptidic vaccine (26–29), or as shown here for HLA-A1 individuals, to raise CTLs in tissue culture that could be used for adoptive immunotherapy (1, 2, 4). Further, these types of cancer treatments could be extended to patients of other frequently found HLA alleles besides HLA-A1, where peptides from the *MAGE-2* and *-3* product sequences that show high-affinity binding to MHC molecules are in the process of being identified.

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