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Generation of specific anti-melanoma reactivity by stimulation of human tumor-infiltrating lymphocytes with MAGE-1 synthetic peptide

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Abstract. The MAGE-1 gene encodes a tumor-specific antigen, MZ2-E, which is recognized by cloned, specific cytolytic T cells (CTL) derived from the peripheral blood of a patient with melanoma. We have produced a MAGE-1-specific CTL line derived from the tumor-infiltrating lymphocytes (TIL) of a melanoma patient by weekly restimulation with autologous EBV-B cells pulsed with the synthetic HLA-A1-restricted MAGE-1 epitope nonapeptide EADPTGHSY. The 1277.A TIL line grew in long-term culture in low-dose interleukin-2 (IL-2) and IL-4, and exhibited antigen-specific, MHC-class-I-restricted lysis of HLA-A1-bearing MAGE-1$^+$ cell lines. Cytolysis of target cells pulsed with the synthetic MAGE-1 decapeptide KEADPTGHSY was superior to that of cells pulsed with the immunodominant nonapeptide. Single amino-acid or even side-chain substitutions in the immunodominant nonamer abrogated cytolysis. 1277.A TIL specifically secreted tumor necrosis factor α after co-incubation with HLA-A1-expressing MAGE-1$^+$ cell lines or fresh tumor. These data suggest that tumor-antigen-specific, MHC-restricted CTL may be grown from TIL in the presence of synthetic epitope peptides and expanded for adoptive immunotherapy in melanoma patients.

Key words: Melanoma – MAGE-1 – Tumor-infiltrating lymphocytes – Immunotherapy – Peptide

Introduction

A variety of murine and human tumors display antigens on their surface in association with MHC class I molecules, which may determine the efficacy of host immune surveillance [19, 27, 35, 36]. The definition and cloning of naturally occurring antigens recognized by the tumor-bearing host is an important factor in stimulating an anti-tumor immune response through immunization, adoptive cell transfer, or other forms of immunotherapy [8]. Cytotoxic T lymphocytes (CTL) can recognize unique [51] or shared melanoma-associated antigens in an HLA-restricted fashion [2, 14, 25]. A number of tumor-associated antigens recognized by CTL have been characterized, including p53 [54], p21ras [41], HPV E6 [18], tyrosinase [7], and members of the MAGE gene family [52]. Enhanced expression of tumor-associated antigens may increase immunogenicity, while the elimination of the genes coding for these antigens can lead to "antigen-loss" variants possessing diminished or absent immunogenicity [15, 51]. Studies by Boon and co-workers have demonstrated that antigen-loss variants of tumor-rejection antigens on the surface of the mouse mastocytoma P815 are resistant to CTL raised against them [4], which could account for their decreased immunogenicity.

Tumor antigens represent potential targets for T-cell-based immunotherapy when expressed in the proper MHC class I context. The importance of tumor antigen expression is underscored by recent work in our laboratory suggesting that down-regulation of antigen processing in association with MHC class I molecules leads to diminished antigen presentation and increased tumor growth [32]. The re-establishment of antigen processing in a deficient murine line by transfection with the interferon γ (IFNγ) gene permitted the generation of tumor-specific T cells [32]. Adoptive transfer of specific populations of cytotoxic T cells directed against tumor-associated antigens presented by MHC class I molecules can result in decreased tumor burden and increased survival in syngeneic hosts [10, 26, 35, 36, 38]. In addition, tumor cells exhibiting low levels of class I expression that evade immune recognition may exhibit enhanced growth [16, 43].

The MAGE-1 gene, encoding the human melanoma-associated antigen MZ2-E, has been cloned and characterized [52]. An immunodominant nonamer peptide presented in the context of HLA-A1 [49] on the surface of

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melanoma and non-melanoma tumor tissue and cell lines is recognized by cytolytic T cells [6, 44, 56]. MAGE-1 is expressed by cell lines and fresh tumor from patients with early, loco-regional, and metastatic melanoma [53]. Tumors expressing MAGE-1 can be specifically lysed by CTL clones generated from a metastatic melanoma patient whose tumor was MAGE-1$^+$, and who had received multiple immunizations with autologous tumor [52]. In this study we report the generation of an MHC-restricted, MAGE-1-reactive CTL line, derived from the tumor-infiltrating lymphocytes (TIL) of a patient with metastatic melanoma prior to immunotherapy. We have developed a method for production of bulk CTL that grow and proliferate in the presence of low-dose interleukin-2 (IL-2) and IL-4 by repeated stimulation of a tumor suspension with autologous EBV-B cell blasts pulsed with the HLA-A1 immunodominant MAGE-1 peptide. These cells are specifically lytic until at least day 96 in culture. This CTL line recognized the MAGE-1 antigen in association with HLA-A1, and could recognize both the natural nonapeptide as well as an overlapping decapeptide with an additional amino-terminal lysine, when they were pulsed onto antigen-presenting cells. The CTL lysed target cells expressing the MAGE-1 nonapeptide, including established tumor cell lines, and immortalized EBV-B lines pulsed with peptide. Yet it did not lyse targets pulsed with a peptide containing amino acid substitutions of the natural nonamer, or even single side-chain substitutions in that peptide sequence. Synthetic peptides derived from immunodominant epitopes of tumor-specific antigens may be useful as vaccines when complexed with adjuvants, and could be used to develop tumor-antigen-specific CTL from tumor suspensions for expansion ex vivo and adoptive immunotherapy.

Materials and methods

Cultured cell lines. All tumor cell lines (with the exception of MZ2-mel.43) were established in the Surgery Branch, National Cancer Institute, as previously described [45] by overnight incubation of minced, fresh tumor with a mixture of 0.1% collagenase type IV, 0.002% DNase type 1 and 0.01% hyaluronidase type V (Sigma Chemical Corp., St. Louis, Mo.) at 25° C. These cell lines have been assigned the suffix "mel". MZ243, which expresses the MAGE-1 gene product MZ2-E in association with HLA-A1, was a kind gift of Dr. Thierry Boon, Ludwig Institute for Cancer Research, Brussels, Belgium [52]. All cultures were maintained as monolayers in RPMI-1640 medium (Biofluids Inc., Rockville, Md.) supplemented with 10% fetal bovine serum, 50 mM HEPES (pH 7.0), 10 nM glutamine, 250 U/ml penicillin/streptomycin, and 0.25 mg/ml gentamicin.

The Daudi lymphoma line served as a lymphokine-activated killer (LAK) cell target, and the K562 erythroleukemia line served as a natural killer cell target, in chromium-release assays.

LAK cells were harvested from normal buffy coats as previously described [34]. They were propagated in RPMI-1640 medium containing the supplements listed above, to which was added 2% human AB serum and 1000 U/ml recombinant (r)IL-2.

Epstein-Barr-virus(EBV)-transformed B cell lines were produced in our laboratory using previously described techniques [45] and maintained as above.

Generation of MAGE-1-specific TIL

On day 0, TIL cultures were established from a single-cell suspension of a solid melanoma metastasis from a patient designated 1277.A. 1277.A TIL were propagated in Iscova's minimal essential medium (IMEM; Biofluids Inc., Rockville, Md.) supplemented with 10% heat-inactivated human AB serum, as well as HEPES, glutamine, penicillin/streptomycin, gentamicin, 20 U/ml rIL-2 (the kind gift of Cetus Corp., Emeryville, Calif.) and 5 U/ml IL-4 (the kind gift of Sterling Drug Inc., Malvern, Pa.). Cultures were set up in 24-well plates at a concentration of 1×10^6 tumor cells/well in 0.5 ml medium. Autologous EBV-B cell stimulators, previously incubated for at least 4 h with 5 μM MAGE-1 peptide with the sequence EADPTGHSY (generously provided by MedImmune Inc., Gaithersburg, Md.), were washed once in IMEM, then irradiated with 30 Gy using a ^{137}Cs source and added to each well in 0.5 ml medium. On day 3, half the medium in each well was replaced by supplemented IMEM containing 20 U/ml rIL-2 and 5 U/ml rIL-4. On day 10, half the medium in each well was replaced by medium (without cytokines) and irradiated stimulators. The following day half the medium per well was replaced by medium plus IL-2 and IL-4. Restimulation followed by addition of cytokines 1 day later was continued weekly. In addition, for the first 10 days, 10% LAK supernatant, prepared as previously described [34], was included in the medium.

Phenotypic analysis. Flow cytometry of 1277.A TIL was performed using a FACScan (Becton Dickinson, Mountain View, Calif.), as previously described [44]. Cells were stained with murine anti-(human Ig) mAbs against CD3 (Leu4), CD4 (Leu3), CD8 (Leu2), CD25 (IL-2Rα), and HLA-DR (anti-DR) (all from Becton Dickinson, Mountain View, Calif.). An isotype-matched murine (IgG2a) antibody served as a negative control.

Peripheral blood mononuclear cells (PBMC). PBMC were obtained from buffy coats or lymphocytopheresis specimens of malignant melanoma patients treated in the Surgery Branch. Cells were isolated by separation over lymphocyte separation medium (Organon-Teknika Corp., Durham, N.C.), and subsequent washing with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution.

HLA typing. HLA typing of peripheral blood lymphocytes (PBL) from tumor-bearing patients and TIL was performed by the NIH HLA Typing Laboratory using the modified Amos microcytotoxicity assay.

RNA and cDNA preparation. Total cellular RNA was obtained from $(1-2)\times10^6$ fresh tumor suspensions or frozen cells, PBL, TIL, or tumor cell lines. Pellets were lysed in a 4 M guanidum isothiocyanate (Sigma Chemical Corp., St. Louis, Mo.) solution, vortexed, then layered over cesium chloride (Gibco BRL, Gaithersburg, Md.). RNA was isolated by centrifugation at 31500 rpm for 15 h using a SW41 swinging-bucket rotor (Beckman Inc., Palo Alto, Calif.). RNA pellets were washed several times with 70% ethanol and recovered by ethanol precipitation. The absorbance ratios (A_{260}/A_{280}) of all preparations were determined with a Beckman DU-65 spectrophotometer (Beckman Inc., Palo Alto, Calif.).

A 2-μg sample of template RNA was brought up to 20 μl with 1× first-strand synthesis buffer, 500 mM deoxynucleotide mix (containing the four deoxynucleotides in equal amounts), 10 μM dithiothreitol, 1 μM dT_{12-18}, 200 U Superscript reverse transcriptase (all from Gibco BRL) and 1 U/ml RNasin (Promega, Madison, Wis.). Following an incubation at 42° C for 40 min, 80 μl sterile distilled water was added to each tube. cDNA was stored at –20° C until use.

Reverse transcriptase/polymerase chain reaction (RT-PCR) amplification of cDNA. RT-PCR amplification of 5 μl cDNA (corresponding to 100 ng RNA) was performed in a 50-μl reaction mix containing 1× Taq reaction buffer, 1.5 mM MgCl2, 25 nM each deoxynucleotide triphosphate, 0.25 μl Taq DNA polymerase (Gibco BRL) and 50 pmol each of sense CHO-14 primer (CGGCCGAAGGAACCTGACCCAG) and antisense CHO-12 primer (GCTGGAACCCTCACTGGGTTGCC), the sequences of which were kindly provided by Dr. T. Boon. CHO-12 binds to a sequence in the first exon of MAGE-1, CHO-14 to a sequence in the open-reading frame of exon 3 [52]. Amplification was carried out for 4 min at 94° C for 1 cycle, followed by 30 cycles of denaturing (94° C, 1 min), annealing and extension

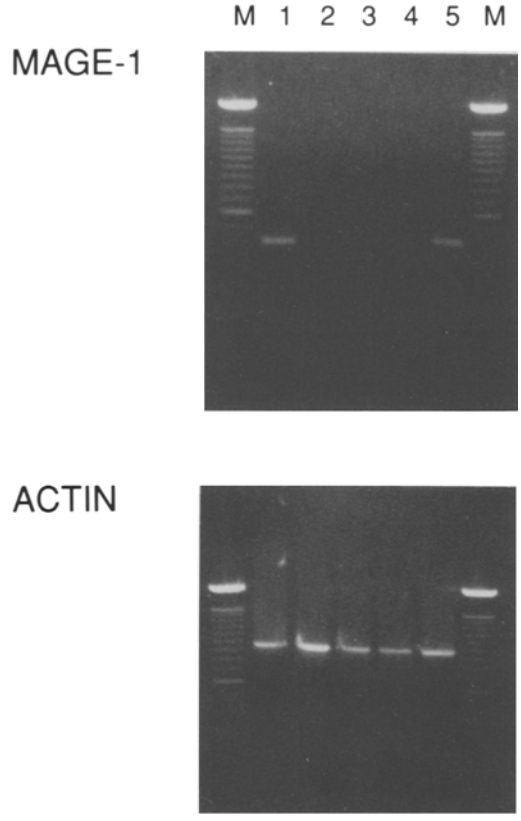

Fig. 1. Reverse transcriptase/polymerase chain reaction (RT-PCR) analysis of MAGE-1 expression in patient 1277.A tumor and normal cells. cDNA was prepared from total RNA as described in Materials and methods. Aliquots were then amplified by PCR using MAGE-1-specific primers (*upper panel*) or actin primers (*lower panel*). Results show MAGE-1 and actin reactions for each sample. PCR products were separated by electrophoresis on 2% agarose gels containing ethidium bromide. *Lane 1*, 1277.A tumor; *lane 2*, 1277.A peripheral blood lymphocytes (PBL); *lane 3*, 1277.A TIL, day 23; *lane 4*, 1277EBV-B; *lane 5*, MZ-243 cultured melanoma cell line. *M*, 100-base-pair ladder

(72° C, 3 min), with a final extension for 15 min at 72° C. RT-PCR was performed using actin primers (Perkin-Elmer Cetus, Emeryville, Calif.) with all RNA samples to ensure that each specimen contained intact RNA. Analysis of PCR products was accomplished by agarose gel electrophoresis.

Cytotoxicity assays. Samples containing 1×10^6 target cells were labelled with 200 μCi ^{51}Cr (New England Nuclear, Boston, Mass.) in 0.5 ml RPMI medium with 2% human AB serum for 2 h at 37° C, then washed several times prior to the addition of cultured TIL or LAK effectors. Targets demonstrating less than 50% viability were centrifuged over a cushion of lymphocyte separation medium to remove dead cells prior to use. Effector and target cells were incubated at 37° C at appropriate E:T ratios for 4 h. Supernatants were collected and counted on a gamma counter. Percentage specific lysis was calculated as: 100× (sample radioactivity – spontaneous radioactivity)/(maximum radioactivity – spontaneous radioactivity). Spontaneous radioactivity (cpm) was obtained from targets incubated with RPMI medium, maximum radioactivity from targets incubated with 2% sodium dodecyl sulfate. All assays were performed in triplicate. Spontaneous ^{51}Cr release did not exceed 25% of the maximum release.

Inhibition of cytotoxicity with mAb. Appropriate targets were incubated with 10 μl preservative-free mAb (31 μg/ml final concentration) for 30 min at room temperature prior to the addition of 1277.A TIL. Antibodies directed against human leukocyte antigen (HLA) determinants were W6/32 (anti-HLA-A, -B, -C shared determinants; Sera-Lab, Sussex, England) and IVA12 (anti-HLA-DR, DP, DQ; purified from ATCC hybridoma supernatant). The final concentration of all antibodies was 30 μg/ml. Negative controls were 10-μl samples of phosphate-buffered saline. Statistical comparisons were performed using Wilcoxon ranked-sum analysis.

Cytokine release. TIL used for cytokine analysis were cultured for at least 3 days without IL-2, then washed twice for further elimination of IL-2 or other cytokines. Samples containing 5×10^5/ml TIL were co-cultured with an equal number of irradiated tumor stimulators in IMEM plus 10% human AB serum for 24 h at 37° C as previously described [20]. Fresh cryopreserved tumors were thawed quickly and rinsed twice to remove dimethylsulfoxide. Cultured cell lines were harvested by exposure to 0.05% trypsin plus 0.02% EDTA (Trypsin/Versene, Biofluids), then washed twice. Supernatants were centrifuged at 2000 rpm to remove cells, then stored at –70° C until use. TIL co-cultured with TNFα-transduced NIH-3T3 fibroblasts served as a positive control for the TNF assay. Targets incubated in the absence of 1277.A TIL served as a measure of spontaneous release.

All cytokine determinations were performed using commercially available enzyme-linked immunoabsorbant assay (ELISA) kits: TNFα (Quantikine Human TNFα Immunoassay, R & D Systems, Minneapolis, Minn.; minimum detectable concentration = 4.8 pg/ml).

Peptides. Peptides were the generous gift of MedImmune Inc. (Gaithersburg, Md.) or Dr. Kari Irvine (National Cancer Institute, Bethesda, Md.). Peptide sequences were as follows: MOO-10, the natural MAGE-1 nonapeptide [48], H_2N-EADPTGHSY-CO_2H; MOO-16, ZADPTGHSY, with a pyroglutamic acid analog (Z) substitution; MOO-19, KEADPTGHSY, the natural MAGE-1 decapeptide; MOO-20, DADPTGHSY, with an asparagine substituted for glutamic acid; MOO-21, E(me)ADPTGHSY, with a *N*-methylated glutamic acid substitution; OVA 256–263, SIINFEKL, an octamer presented by the EL4 tumor ($H\text{-}2^b$-restricted) when transfected with the ovalbumin gene [30]; P1A, LPYLGWLVG, the immunodominant nonamer presented by the mouse mastocytoma line p815 and restricted by L^d [51]; ES-NP 147–155, containing an endoplasmic-reticulum-signal sequence (TYQRTRALV) ligated to the influenza A/PR/8/34 nucleoprotein immunodominant nonamer [30] (and unpublished data).

Results

Identification of MAGE-1 expression by donor tissue and cells

Total cellular RNA was extracted from single-cell suspensions of metastatic melanomas. Following cDNA synthesis, RT-PCR was performed using intron-spanning primers specific for the first and third exons of the MAGE-1 gene. Specimens expressing the MAGE-1 gene product produce a 420-bp fragment, readily discernible from the PCR fragment greater than 2 kb that would result from any DNA contamination (T. Boon, personal communication). Figure 1 shows the MAGE-1 RT-PCR analysis of cells obtained from patient 1277.A. The 420-bp PCR product of the MAGE-1 gene was only generated from tumor RNA (lane 1). MAGE-1 was not expressed by autologous PBL (lane 2), TIL (lane 3), or the EBV-B cell line (lane 4). MAGE-1 was expressed by the established cell line, MZ-243 (lane 5), which expresses the MAGE-1 gene product MZ2-E in association with HLA-A1 [52]. RT-PCR results when actin primers were used demonstrated that intact RNA was used in each sample (lower panel). Therefore, MAGE-1 was

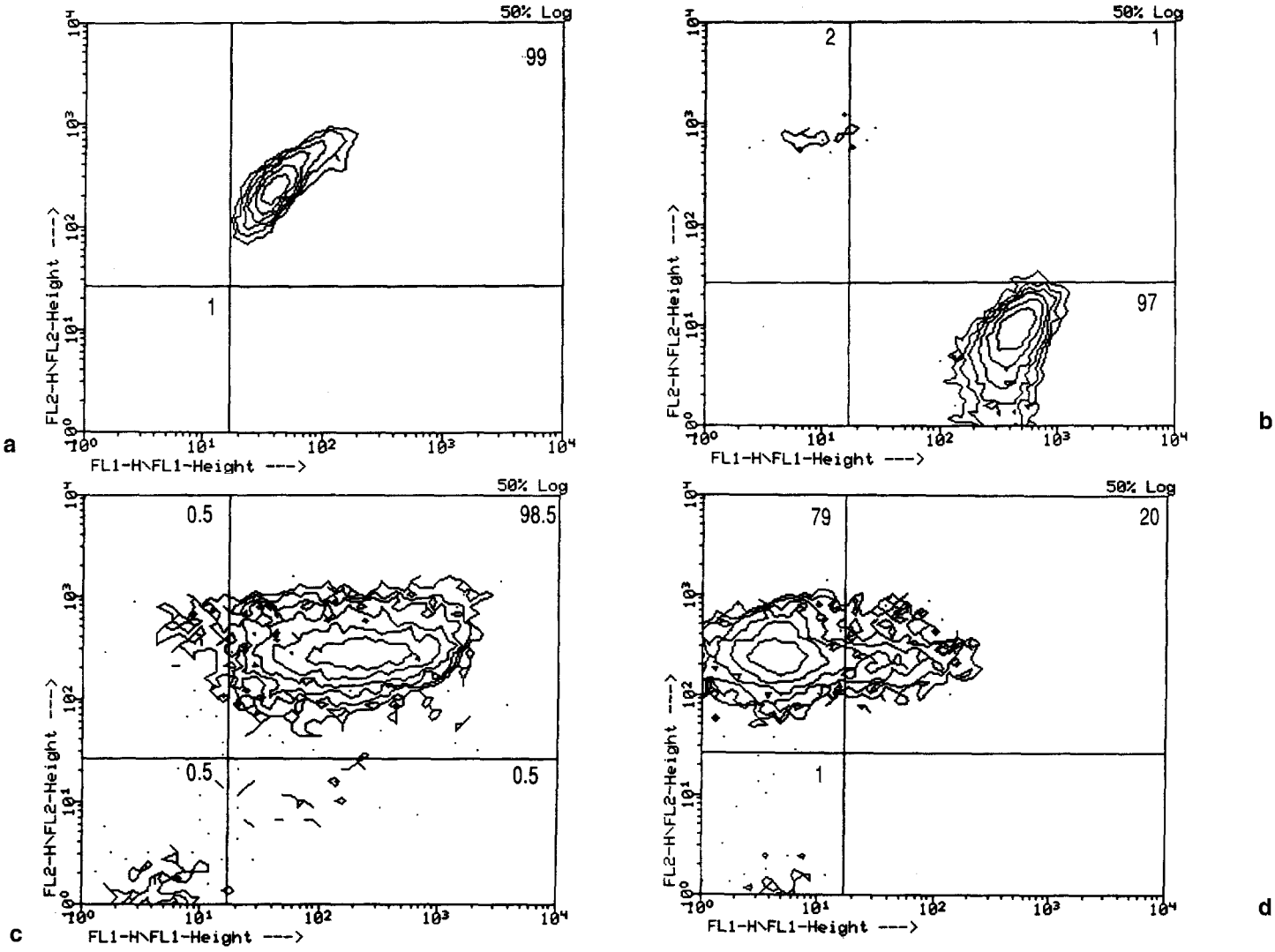

Fig. 2a–d. Two-color immunofluorescence analysis of 1277.A TIL. Antibody staining for flow cytometry was as described in Materials and methods. **a** CD3 staining indicated on the ordinate, T cell receptor αβ on the abscissa; **b** CD4 staining indicated on the ordinate, CD8 on the abscissa; **c** CD3 staining indicated on the ordinate, HLA-DR on the abscissa; **d** CD3 staining indicated on the ordinate, CD25 on the abscissa. Percentage positive cells in each quadrant are indicated

expressed by the tumor cells, but not by normal or EBV-transformed cells from patient 1277.A.

Growth kinetics and characterization of 1277.A TIL from tumor suspension

At day 0, frozen tumor cells from patient 1277.A were thawed, counted, and plated in 24-well dishes at a concentration of 0.5×10^5 cells/well. Approximately 30% of the population were tumor cells and had 50% viability by light-microscopic examination and trypan blue exclusion. Cells were plated in 2 ml medium containing 10% human AB serum and 10% LAK supernatant. On day 3, half the volume was replaced with medium containing 20 U/ml rIL-2 and 5 U/ml rIL-4. Restimulation with 1×10^6/well irradiated autologous EBV-B cells incubated with the MAGE-1 peptide was repeated weekly for 96 days. TIL had a doubling time of 2–3 days until approximately day 60 at which time their growth slowed, and they became quiescent around day 82. Cells remained over 80% viable at day 96 (data not shown). Growth characteristics of a second independent generation of MAGE-1-specific 1277.A TIL were nearly identical (data not shown).

Flow-cytometric analysis of 1277.A TIL was performed on day 68 (Fig. 2). Over 98% of the population was positive for T cell receptor αβ (TCRαβ+) and more than 97% of those cells were CD8+ (Fig. 2a, b). Activation markers were expressed by 97.1% of cells, as shown by anti-HLA-DR staining (Fig. 2c), and 19.6% also stained for expression of the p55 IL-2α low-affinity receptor (Fig. 2d). These data suggest that 1277.A TIL were predominantly CD3+/CD8+ activated TCRα/β-expressing T cells.

1277.A TIL demonstrate HLA-A1-restricted lysis of targets expressing MAGE-1

At various times after the initiation of the culture, the cytolysis of effector 1277.A TIL stimulated with MAGE-1

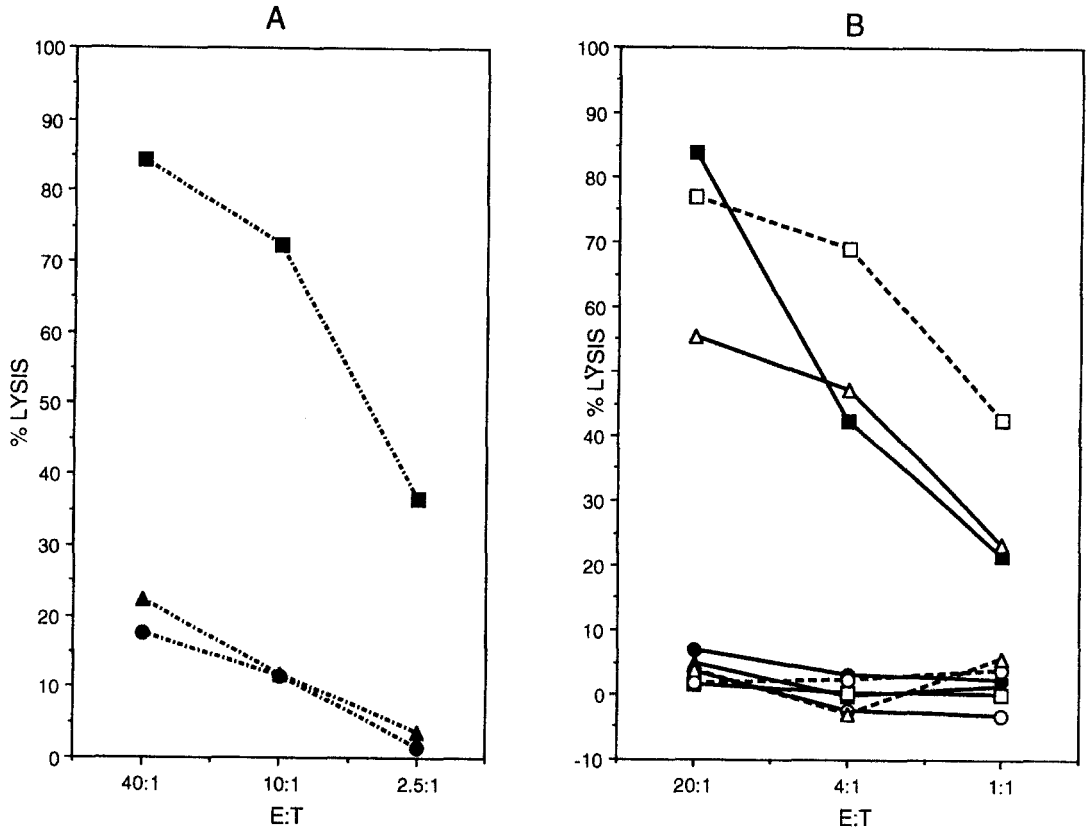

Fig. 3 A, B. Lysis of peptide-pulsed autologous EBV-B cells and HLA-A1+, MAGE-1+ cell lines by 1277.A TIL. ^{51}Cr-release assays were performed with 1277.A TIL effectors using either autologous EBV-B cells with and without peptide (5 μM final concentration incubated at 37° C overnight), tumor cells lines (397mel, 501mel, 586mel, 624mel, 888mel, 938mel, MZ243, Daudi), or normal foreskin melanocytes. **A** Lysis of targets by 1277.A TIL at day 42 following five rounds of stimulation. **B** Cytotoxicity using 1277.A TIL at day 68. Targets were 1277EBV-B cells + MAGE-1 (-·■·-); 1277EBV-B + P1A (-·▲·-); 1277EBV-B (-·●·-); 397mel (□) and 938mel (△) (both HLA-A1, MAGE-1+); 501mel (○), 586mel (-·▲·-) and 624mel (□) (all non-HLA-A1, MAGE-1+); 888mel (-·●·-) (HLA-A1, MAGE-1-); MZ243 (---□---) (HLA-A1, MAGE-1+); foreskin melanocytes (---○---); Daudi cells (---△---). Results of a second assay were similar

peptide was tested against EBV-B targets pulsed with various peptides in a 4-h chromium-release assay. In the earliest experiments (day 42 in culture) autologous EBV-B cells alone or pulsed with relevant or irrelevant (P1A) peptide were tested as targets (Fig. 3a). By day 42, following five rounds of stimulation, 1277.A TIL demonstrated more than 80%, lysis of autologous EBV-B cells pulsed with the MAGE-1 peptide, compared with only 20% lysis observed against autologous EBV-B cells alone or pulsed with P1A, at an E:T of 40:1 (Fig. 3a). During subsequent weeks of stimulation, MAGE-1-specific lysis reamined high as non-specific lysis continued to decline. A chromium-release assay performed on day 68 (Fig. 3b) showed excellent lysis against MZ243 (78%, at an E:T ratio of 20:1), a clone of a tumor cell line used to derive anti-MAGE-1 CTL [52]. 1277.A TIL also demonstrated strong lysis of 397mel and 938mel, two additional HLA-A1+, MAGE-1+ cell lines. Lysis was minimal (never greater than 9% at any E:T ratio) against three non-HLA-A1, MAGE-1+ cell lines (501mel, 586mel, and 624mel), 888mel (HLA-A1 but MAGE-1-), or Daudi (the non-specific control for LAK activity). Autologous EBV-B cells alone or pulsed with the irrelevant P1A peptide were again lysed significantly less than when pulsed with MAGE-1 (data not shown). Cultured HLA-A1+ normal melanocytes demonstrated minimal lysis (below 5%). Results were similar when the generation of 1277.A TIL (from an identical aliquot of frozen tumor suspension) was repeated (data not shown).

In order to establish further that 1277.A TIL recognize MAGE-1 in the context of HLA-A1, a chromium-release assay was carried out using a variety of HLA-A1+ and HLA-A1- EBV-B cell lines pulsed with the MAGE-1 peptide (Fig. 4). Only EBV-B cells derived from patients 1328 (A1, 11) and 1088 (A1, 2) pulsed with the MAGE-1 peptide were able to produce a strong CTL response. EBV-B cells derived from patient 501 (A2, 3) and pulsed with the MAGE-1 peptide were minimally lysed (less than 5%). Those three patients did not share any known HLA-B, C, or DR determinants (data not shown), so restriction by other alleles was unlikely. Lysis by 1277.A TIL of unpulsed targets or targets expressing P1A was less than 10%. In duplicate cytolytic assays, K562 cells (a natural killer target) were lysed less than 10% by 1277.A TIL at every E:T ratio (data not shown).

To determine whether 1277.A TIL recognized MAGE-1 expressing targets in an MHC-restricted manner, mAb generated against MHC molecules were utilized to block 1277.A TIL cytolysis (Fig. 5). 1277.A TIL reactivity against MAGE-1 was significantly eliminated by the anti-(class I)

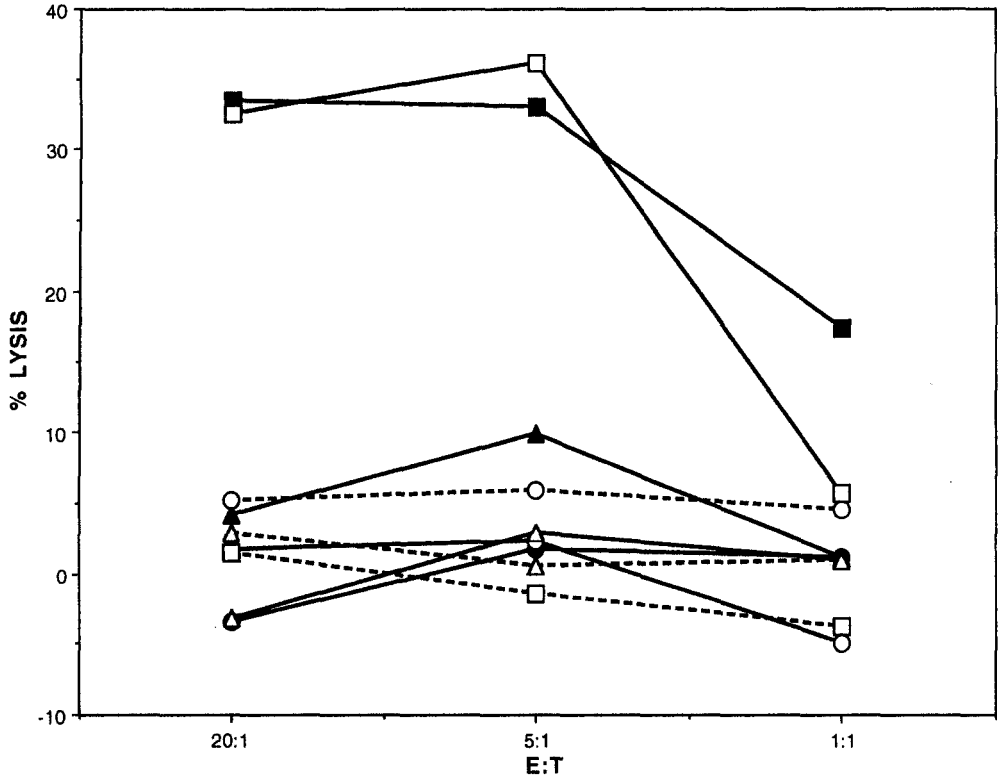

Fig. 4. Cytolysis of targets by 1277.A TIL is restricted by the HLA-A1 haplotype. ^{51}Cr-labeled EBV-B target cells were incubated for 4 h with and without 5 μM MAGE-1 peptide. 1328EBV-B was derived from a patient expressing HLA-A1,11, 1088EBV-B from a patient expressing HLA-A1,2, and 501EBV-B from a patient expressing HLA-A2,3. P1A is the immunodominant P815 nonamer restricted by L^d. Targets were 1328EBV-B cells + MAGE-1 (■); 1328EBV-B + P1A (●); 1328EBV-B (▲); 1088EBV-B + MAGE-1 (□); 1088EBV-B + P1A (○); 1088EBV-B (△); 501EBV-B cells + MAGE-1 (–□–); 501EBV-B + P1A (–○–); 501EBV-B (–△–). Results of a second assay were similar. Day 61 CTL were used in the first experiment, and day 68 cells subsequently

mAb W6/32 yet only slightly diminished by the anti-(class II) mAb IVA12. A 63% inhibition of lysis against the autologous EBV-B cells pulsed with MAGE-1 peptide was observed with W6/32, compared to 20% with IVA12 (Fig. 5a). When two A1+, MAGE-1+ cell lines were used as targets, 62% inhibition was observed with W6/32, and 24% with IVA12 (Fig. 5b, c). The percentage inhibition for each target was significantly different compared with controls at a *P* value below 0.05. The percentage inhibition of lysis was minimal when phosphate-buffered saline was used as a negative control. These data suggest that 1277.A TIL lyse MAGE-1-expressing targets largely in a class-I-MHC-dependent manner, restricted by HLA-A1.

Modifications of the MAGE-1 peptide alter cytotoxicity by 1277.A TIL

Since single amino-acid substitutions in epitope sequences have been shown either to abolish recognition by CTL [40] or, in some cases, to generate more antigenic peptides, we tested 1277.A TIL cytolysis of autologous EBV-B cells pulsed with various modifications of the MAGE-1 nonamer EADPTGHSY (MOO-10). The results shown in Fig. 6 indicate that the MOO-19 peptide, consisting of the naturally occurring decamer (KEADPTGHSY), produced greater lysis by 1277.A TIL than did MOO-10 (the natural MAGE-1 nonamer) when they were pulsed onto autologous EBV-B cells. 1277.A TIL lysis of autologous EBV-B cell targets pulsed with MOO-19 was more than 80%, as compared to 60% lysis of autologous EBV-B cells pulsed with MOO-10, at E:T ratios ranging from 40:1 to 2.5:1. A single amino-acid substitution of aspargine for glutamic acid (MOO-20, DADPTGHSY) largely abolished lysis by 1277.A TIL. EBV-B cells pulsed with synthetic nonamers containing analog substitutions for the amino-terminal peptide – MOO-16, with a pyroglutamic acid substitution, and MOO-21, with a *N*-methylated glutamic acid substitution – were not lysed. When this experiment was repeated to include an E:T ratio of 1:1, results were similar and titration of the lysis curve was observed (data not shown).

Therefore, we used two highly reactive peptides (MOO-19 and MOO-10) and one poorly reactive peptide (MOO-16, as a negative control) in a peptide titration analysis with 1277.A TIL (Fig. 7). In repeated experiments, greater lysis of peptide-pulsed EBV-B targets was obtained with MOO-19 than MOO-10 over a variation of several log units in concentration from 10 nM to 300 nM. Our findings suggest that the addition of the amino-terminal lysine in the MOO-19 decamer stabilizes its interaction with MHC class I and the T cell receptor molecules. Autologous EBV-B cells pulsed with control peptide MOO-16 displayed less than 5% lysis by 1277.A TIL at peptide concentrations up to 10 μM.

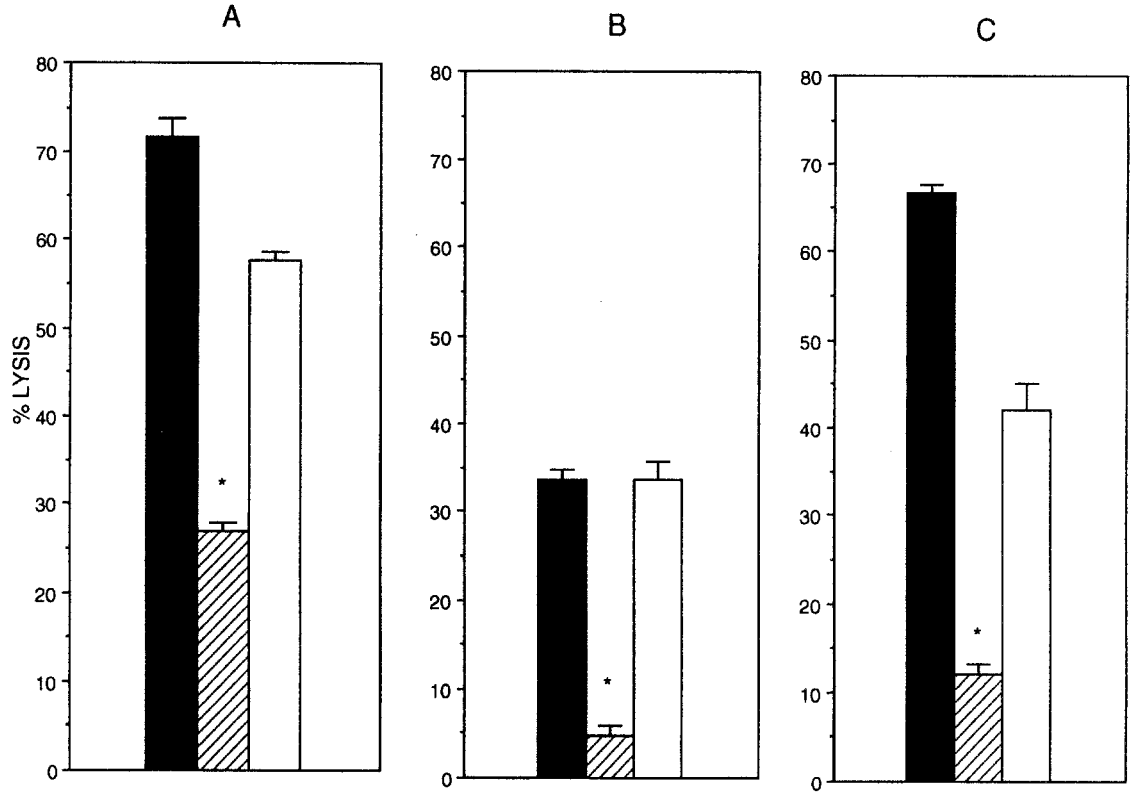

Fig. 5A–C. Inhibition of 1277.A TIL lysis by anti-MHC class I antibodies. Samples containing 5×10^3 target cells were incubated for 30 min with 30 μg/ml antibody in 10 μl prior to addition of effectors. W6/32 (*hatched columns*) was the framework anti-(class I) mAb, IVA12 (*white columns*) the anti-(class II) mAb, and phosphate-buffered saline (*black columns*) was the negative control. **A** Target was 1277EBV-B cells pulsed with the MAGE-1 peptide. **B** Target was 397mel, a HLA-A1/MAGE-1+ established cell line. **C** Target was MZ243, a HLA-A1/MAGE-1+ established cell line. *, A significant difference from the negative control as determined by Wilcoxon ranked-sum test, $P < 0.05$. *Error bars* represent standard error for each group run in triplicate. Results obtained with an E:T ratio of 1:1 are shown. 1277.A TIL were at day 75 of culture in this experiment, and results of a second assay (day 82) were similar

Table 1. 1277.A tumor-infiltrating lymphocytes (TIL) exhibit MAGE-1-specific tumor necrosis factor α (TNFα) secretion

Melanoma (stimulator/target)	TNFα (pg/ml^{-1} 10^6 cells^{-1})		
	Expt. 1[a]	Expt. 2[a]	Targets without TIL co-incubation
TNFα-transduced NIH-3T3	485.7	196.1	–
1277.A TIL alone	<4.8[d]	<4.8	–
+ 1277 tumor (A1/M+)[c]	377.9	132.8	40.2
+ 1337 tumor (A1/M+)	189.1	98.5	27.9
+ 1292 tumor (non-A1/M+)	58.6	13.8	106.4
+ 1329 tumor (A1/M-)	35.9	30.0	74.3
+ 397mel[b] (A1/M+)	23.8	30.1	<4.8
+938mel (A1/M+)	198.6	98.7	<4.8
+ 526mel (A2/M+)	<4.8	<4.8	<4.8
+624mel (A2/M+)	<4.8	<4.8	<4.8
+ 1277 EBV-B	19.5	13.0	–
+ 1277 EBV-B + MAGE-1	55.7	33.2	–
+ 1277 EBV-V + OVA	9.4	6.9	–

[a] Measurement of TNFα in supernatants from 1277.A TIL/tumor co-incubations, as determined by specific double-determinant enzyme-linked immunosorbent assay. Cell-free supernatants were collected 24 h after CTL were co-cultured in duplicate with fresh tumor, EBV-B cells, or cultured tumor cell lines. Separate supernatant harvests were used in experiments 1 and 2. Only experiment 2 was performed concurrently with control supernatants from targets without TIL co-incubation

[b] Established melanoma cell lines are indicated by the suffix "mel"

[c] Fresh tumors and cell lines from HLA-A1-positive patients are labeled A1; those with any other allele are labeled non-A1. M+ indicates MAGE-1 expression and M- the lack thereof, as determined by reverse transcriptase/polymerase chain reaction

[d] Detection limit of assay

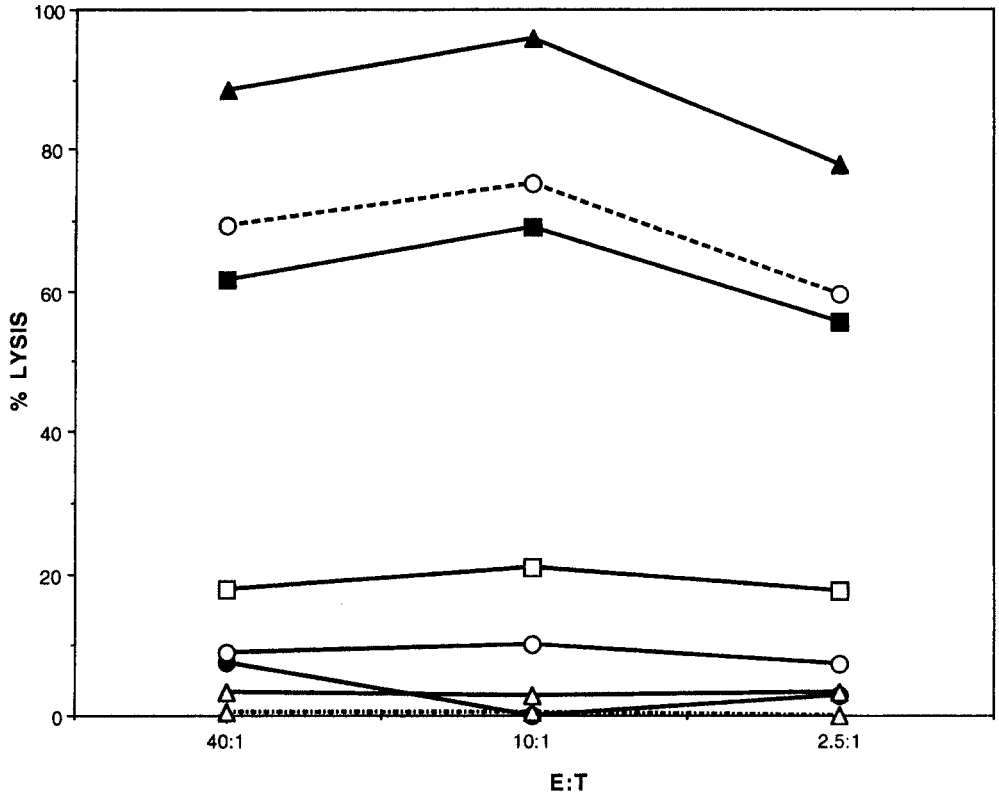

Fig. 6. Comparison of normal EBV-B cells and cells pulsed with mutated MAGE-1 peptide as targets for 1277.A TIL (day 89). EBV-B cells were pulsed with peptide as described in Materials and methods. Sequences are as follows: MOO-10 (■), the naturally occurring nonamer (EADPTGHSY); MOO-16 (●), containing a pyroglutamic acid substitution (ZADPTGHSY); MOO-19 (▲), the naturally occurring decamer (KEADPTGHSY); MOO-20 (□), containing a glutamic acid/asparagine substitution (DADPTGHSY); MOO-21 (○), with *N*-methylated glutamic acid substitution [E(Me)ADPTGHSY]. 938mel (–○–), a MAGE-1-expressing melanoma cell line from an HLA-A1 patient served as positive control. 1277EBV-B (△) and Daudi (–△–) served as non-specific targets. Results of one representative experiment of two are shown

1277.A TIL exhibit MAGE-1-specific cytokine secretion

Since cytolytic T cells often elicit antigen-specific secretion of cytokines such as granulocyte/macrophage-cloning-stimulating factor (GM-CSF) and tumor necrosis factor α (TNFα) following co-incubation with target cells [39], we tested whether 1277.A TIL secreted cytokines after incubation with various MAGE-1-expressing established cell lines and fresh tumor cells (Table 1). TIL co-cultured for 24 h with autologous MAGE-1+ tumor cells secreted significant amounts of TNFα (377.9 and 288.2 pg/ml) in two separate experiments. 1277.A TIL also secreted high amounts TNFα (189.1 and 101.8 pg/ml; $P < 0.05$) when stimulated with another HLA-A1+ tumor (tumor 1337). Both HLA-A1+, MAGE-1+ tumors (1277 and 1337) stimulated significantly more TNFα secretion by 1277.A TIL ($P < 0.05$) compared to the amount released when 1277.A TIL were stimulated with an HLA-A1+, MAGE-1− tumor (no. 1329: 35.9 and 30.0 pg/ml) or a tumor that was HLA-A1− (no. 1292: 58.6 and 13.8 pg/ml). Although the level of TNFα release by 1277.A TIL following incubation with tumors 1329 and 1292 was elevated, it was in fact less than the levels of cytokine released by these tumors alone (74.3 and 106.4 pg/ml respectively). Autologous EBV-B cells pulsed with the MAGE-1 nonamer elicited significantly more TNFα (55.7 and 33.2 pg/ml) than EBV-B alone (19.5 and 13.0 pg/ml) or pulsed with OVA peptide (9.4 and 6.9 pg/ml; $P < 0.05$). Also, incubation of 1277.A TIL with two HLA-A1+, MAGE-1+ cell lines elicited significantly more TNFα (397mel: 23.8 and 30.1 pg/ml; 938mel: 198.6 and 98.7 pg/ml) than incubation with two HLA-A1− cell lines (562mel: <4.8 and 6.9 pg/ml; 624mel, <4.8 pg/ml) with a P value of less than 0.01. Neither 1277.A TIL incubated without stimulators nor targets alone produced measurable levels of cytokines. Our findings indicate that 1277.A TIL specifically secrete TNFα after incubation with MAGE-1-expressing, HLA-A1-bearing cells.

Discussion

CTL can recognize peptides degraded from intracellular proteins and presented on the cell surface complexed with appropriate MHC class I molecules [17, 46, 47]. CTL with unique specificity have been generated against a variety of tumor-associated molecules [1, 7, 51]. In particular, both unique and shared melanoma antigens have been shown to

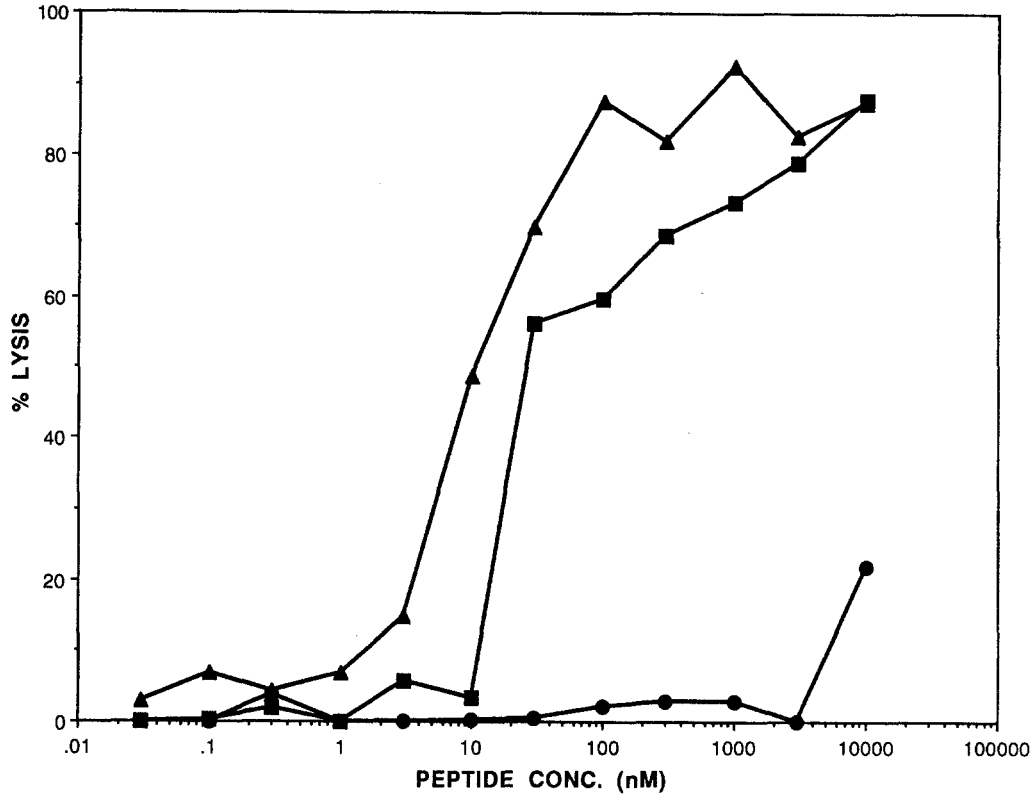

Fig. 7. Lysis by 1277.A TIL of autologous EBV-B cells pulsed with increasing concentrations of natural or mutated MAGE-1 peptides. All incubations with peptide were performed overnight at 37° C at the various concentrations indicated. Sequences were the same as in Fig. 6: MOO-10 (■), the naturally occurring nonamer (EADPTGHSY); MOO-16 (●), containing a pyroglutamic acid substitution (ZADPTGHSY); MOO-19 (▲), the naturally occurring decamer (KEADPTGHSY). 1277.A TIL were used at day 23 of culture

stimulate recognition by MHC-restricted CTL [2, 14, 45]. Point mutations of tumor-associated genes such as the mouse mastocytoma P198 [40] or human *ras* genes [41] can produce potent novel peptide epitopes that bind efficiently to the MHC class I molecule and stimulate recognition by CTL [5]. Three naturally expressed genes have been described that encode antigens that elicit tumor-associated CTL responses: P1A, the gene expressed by mouse mastocytoma P815 [51], tyrosinase [7], and MAGE-1 [49, 52]. Of these, MAGE-1 expression is confined almost exclusively to tumor tissue, the exception being normal testis [5, 53]. Thus, its gene product is an attractive candidate antigen for human adoptive immunotherapy.

One problem in the study of the MAGE-1 gene was the difficulty in obtaining CTL reacting with the MAGE-1 gene product. The only CTL reactive against MAGE-1 was a tumor-specific CTL line derived from PBL of a patient that was rendered free of disease, immunized with autologous tumor cells, and repeatedly stimulated in vitro with autologous fresh tumor [52]. The present investigation showed that MAGE-1-specific CTL can be generated from TIL derived from fresh tumor following repeated in vitro stimulation with antigen-presenting cells pulsed with the immunodominant synthetic nonamer, similar to methods previously used to generate $CD8^+$ HLA-class-I-restricted CTL directed against viral epitopes [9, 50]. We have shown that MAGE-1-specific CTL can be generated from a tumor-bearing patient prior to any immunotherapy.

We used EBV-B as antigen-presenting cells instead of fresh tumor or peptide-pulsed PBL to provide a long-term, autologous stimulator source. B lymphocytes have been shown to function as antigen-presenting cells for MHC-class-I- and -II-restricted antigens [11, 12, 23] including tumor antigen from whole melanoma cell lysates[1]. Adding peptide-pulsed autologous EBV-B cells as stimulators without feeder cells could circumvent the elevation of NK-associated reactivity that Coulie et al. [13] encountered when autologous PBL were used as feeder cells to generate MAGE-1-specific pCTL. Also, we supplemented the medium with low-dose IL-4 on the basis of the observation that it promoted proliferation of responder lymphocytes in mixed lymphocyte culture [13] and induced active CTL from $CD4^-CD8^+$ pCTL [29].

The CTL line that was produced, 1277.A, grew for over 3 months in tissue culture and demonstrated specific long-term cytolysis of HLA-A1-restricted, MAGE-1^+ targets including established melanoma cell cultures, fresh melanomas, and allogenic EBV-B cells pulsed with MAGE-1. With a 2–3 day doubling time, 10^{10}–10^{11} cells could be generated within 4–6 weeks, the therapeutic titer and time

[1] Topalian, S. L., Mancini, M., Rivoltini, L., Ng, J., Hatrzman, R. J., Rosenberg, S. A. Melanoma-specific $CD4^+$ lymphocytes recognize human melanoma antigens processed and presented by Epstein-Barr virus transformed B cells. Unpublished results

frame necessary for effective treatment. 1277.A TIL predominantly exhibited a $CD3^+CD8^+$ phenotype and expressed TCRα/β. It demonstrated extremely low cytotoxicity against normal fibroblasts or the LAK and NK control cell lines Daudi and K562. Studies are underway to determine its activity against non-melanoma targets. We are also attempting to repeat our experiments with PBL or TIL from other patients, and preliminary results indicate the generation of MAGE-1-specific CTL from one of three additional patients studied (J. Weber, unpublished data).

We are certain that the particular pulsed peptide (EADPTGHSY) on the stimulator cells uniquely directed the generation and expansion of our MHC-restricted, MAGE-1-specific CTL. At least two peptides that encode potential epitopes from the MAGE-3 molecule (E. Celis, personal communication) failed to induce the production of MAGE-1-specific CTL when pulsed onto 1277EBV-B cells and used as stimulators to expand in parallel an aliquot of TIL from patient 1277. In addition, 1277.A TIL never displayed more than 10% cytolysis of MAGE-3A- or MAGE-3B-pulsed targets (data not shown).

Cytolysis by 1277.A TIL was high against established cell lines. Moreover, specific secretion of TNFα was observed following co-cultivation of 1277.A TIL with fresh autologous and other $HLA\text{-}A1^+$ fresh tumor and established cell lines. Thus, 1277.A TIL specifically recognized MAGE-1-expressing fresh tumor cells and established cell culture lines. The importance of cytokine secretion by tumor-specific CTL was underscored by the previous studies in our laboratory demonstrating that TIL generated by conventional techniques specifically released IFNγ and TNFα following co-cultivation with autologous and other HLA-matched fresh tumors [3, 20, 39]. It is important to note that the therapeutic effectiveness of several TIL cultures correlated better with their capacity to secrete TNFα specifically than with their cytotoxic ability in vitro [3]. TNFα secretion by CTL has also been used to help identify the tumor antigens MAGE-1 and tyrosinase [6, 52].

Preliminary studies have assessed whether 1277.A TIL demonstrate cytolysis of thawed, frozen melanoma cells derived from enzyme-digested single-cell suspensions of fresh tissue. Two tumors expressing MAGE-1 and derived from HLA-A1 patients were more than 13% lysed by 1277.A TIL, while none of the two MAGE-1-expressing tumors from non-HLA-A1 patients or three samples from HLA-A1 patients whose tumors did not express MAGE-1 were lysed more than 4% by 1277.A TIL (data not shown). Further experiments will be required to determine to what extent 1277.A TIL can uniquely recognize MAGE-1-expressing fresh tumor cells derived from melanomas. Low MHC class I expression by tumors in vivo, which can correlate with diminished immunogenicity in vivo [2, 16, 31], can produce poor cytolysis of fresh tumor targets. It would be useful to determine if our fresh tumor targets expressed the threshold level of HLA-A1 molecules on the cell surface thought to be necessary to induce antigen-specific CTL responses [16].

We were unsuccessful at generating MAGE-1-specific CTL from three fresh tumors (including patient 1277) that were first stimulated with peptide-pulsed antigen-presenting cells 2 or 3 weeks after initiation of culture (M. Salgaller, unpublished data). It is likely that, unless peptide stimulation begins soon after the in vitro sensitization is established, it becomes difficult to elicit proliferation of the relevant CTL subpopulation from the heterologous effector population in the tumor infiltrate. To improve further the generation of tumor antigen-specific CTL for immunotherapy, studies are underway to assess the stimulatory requirements for eliciting MAGE-1-specific CTL from TIL or PBL, and to test whether dendritic cells or elutriated monocytes could serve as antigen-presenting cells in this system [22].

When the sequence of viral or tumor antigens is known, synthetic peptides, such as the one used in this report, can be used to generate CTL by pulsing the appropriate peptide onto antigen-presenting cells to generate and expand MHC-restricted CTL for adoptive immunotherapy. Also, once identified, the immunodominant epitope might be altered to increase its immunogenicity [5, 54]. For example, the overlapping naturally occurring MAGE-1 decamer (KEADPTGHSY) elicited greater lysis than the natural nonamer (EADPTGHSY) when pulsed onto autologous EBV-B cells, even at tenfold lower concentrations. In a titration analysis, the concentration of the MAGE-1 decapeptide required for maximal cytolysis was comparable to results obtained with the more immunogenic tum- transplantation antigen, P91A [28], or the influenza NP peptide [37]. Increased HLA-peptide complex stability and density of the pulsed peptide on the presenting cell surface may facilitate a strong antitumor immune response.

At present only about 10% of melanoma patients could benefit from MAGE-1-based immunotherapy since only about 25% of the United States population is $HLA\text{-}A1^+$ and only 40% of melanomas express MAGE-1. Therefore, studies are underway to generate CTL against MAGE-3, expressed in almost 80% of melanomas (J. Weber, unpublished results), and to find human melanoma-associated antigens presented by other HLA phenotypes. It is important to study putative tumor-associated antigen peptides presented by other haplotypes since MHC haplotype restriction limits the response within a given population of patients [21].

Numerous studies have confirmed the immunological reactivity of CTL raised against defined antigens [2, 9, 24, 50, 55]. In animal models, adoptive transfer of T cells generated against defined viral or tumor antigens was therapeutically effective [18]. In humans, when PBL without tumor specificity from patients with advanced melanoma were co-cultured in vitro with autologous tumor cells and IL-2 and adoptively transferred to the patient, no therapeutic response was observed [42]. However, cyclomegalovirus(CMV-specific $CD8^+$ CTL, generated from CMV-seropositive bone marrow donors and adoptively transferred to immunodeficient allogeneic bone marrow transplant recipients, selectively restored CMV immunity for over 4 weeks without toxicity [33]. Tumor-specific TIL derived from fresh tumor digests of melanoma patients can mediate significant regression of disease when adoptively transferred with IL-2 [35]. The definition of specific tumor antigens from a variety of malignancies should facilitate the growth of highly tumor-reactive CTL by methods described in this study. These findings emphasize the poten-

tial therapeutic benefit of tumor-antigen-specific T cells, such as those generated in our study, for the persistent enhancement of antitumor immunity in tumor-bearing patients who receive adoptively transferred cells.

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