# Identification of Five MAGE-A1 Epitopes Recognized by Cytolytic T Lymphocytes Obtained by In Vitro Stimulation with Dendritic Cells Transduced with *MAGE-A1*[1]

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***MAGE*** **genes are expressed by many human tumors of different histological types but not by normal cells, except for male germline cells. The Ags encoded by** ***MAGE*** **genes and recognized by T cells are therefore strictly tumor-specific. Clinical trials involving therapeutic vaccination of cancer patients with MAGE antigenic peptides or proteins are in progress. To increase the range of patients eligible for therapy with peptides, it is important to identify additional MAGE epitopes recognized by CTL. Candidate peptides known to bind to a given HLA have been used to stimulate T lymphocytes in vitro. In some instances, CTL clones directed against these synthetic peptides have been obtained, but these clones often failed to recognize tumor cells expressing the relevant gene. Therefore, we designed a method to identify CTL epitopes that selects naturally processed peptides. Monocyte-derived dendritic cells infected with a recombinant canarypoxvirus (ALVAC) containing the entire** ***MAGE-A1*** **gene were used to stimulate CD8$^+$ T lymphocytes from the blood of individuals without cancer. Responder cell microcultures that specifically lysed autologous cells expressing MAGE-A1 were cloned using autologous stimulator cells either transduced with a retrovirus coding for MAGE-A1 or infected with recombinant** ***Yersinia*****-MAGE-A1 bacteria. The CTL clones were tested for their ability to lyse autologous cells loaded with each of a set of overlapping MAGE-A1 peptides. This strategy led to the identification of five new MAGE-A1 epitopes recognized by CTL clones on HLA-A3, -A28, -B53, -Cw2, and -Cw3 molecules. All of these CTL clones recognized target cells expressing gene** ***MAGE-A1*****.** ***The Journal of Immunology,*** **1999, 163: 2928–2936.**

Cytolytic T lymphocytes that lyse human tumor cells have been isolated frequently from cultures of irradiated tumor cells and autologous lymphocytes (1, 2). Some of these CTL have been used as tools to isolate the genes that code for the tumor Ags, such as those of the *MAGE* gene family, which includes at least 17 genes (3–7). The *MAGE* genes are expressed by tumors of different histological types, but they are silent in normal cells, with the exception of male germline cells that do not express HLA class I molecules and are therefore unable to present Ags to CTL (8, 9). Because the MAGE Ags are shared by many tumors and because of their strict tumoral specificity, they are of particular interest for cancer immunotherapy.

Recently, numerous *MAGE*-type genes (i.e., genes that are expressed in tumors and not in normal cells except in male germline cells) have been isolated either by purely genetic approaches or with Abs present in sera of cancer patients (4, 7, 10–12). As a result, a large number of new sequences coding for potential tumor Ags recognized by CTL are now available, but the Ags are identified at a much slower rate. We have therefore tried to devise general approaches for the identification of new antigenic peptides on the basis of available coding sequences.

Our first approach was based on the in vitro stimulation of T lymphocytes with candidate peptides previously shown to be good binders to a given HLA molecule. Although straightforward and sometimes successful (13–18), this approach presents a major drawback: many peptide-specific CTL do not recognize HLA-matched tumor cells expressing the protein endogenously, presumably because the selected peptide is not generated efficiently by the processing machinery of the cell or because the CTL obtained after stimulation with high concentrations of peptide have a low affinity (19). Therefore, we attempted to stimulate T lymphocytes with dendritic cells transduced with a *MAGE* gene. Because transfection of *MAGE-A1* into dendritic cells by lipofection, electroporation, or "gene-gun" was unsuccessful, we used the ALVAC delivery vector (20). ALVAC, a canarypoxvirus, has been shown to infect mammalian cells in vitro as readily as avian cells, its natural host cells. But in mammalian cells, its genome does not replicate and no progeny virus is produced. Blood mononuclear cells infected with a recombinant ALVAC vector have been shown to activate and expand specific CTL precursors from the blood of HIV-positive donors (21). An anti-MAGE-A1 lytic activity was also reported after stimulation of lymphocytes infiltrating a *MAGE-A1*$^+$ tumor with blood mononuclear cells infected with an ALVAC containing *MAGE-A1* (22).

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We report in this paper that anti-MAGE CTL clones were obtained by stimulating T lymphocytes with dendritic cells infected with ALVAC-MAGE-A1. This led to the identification of five new MAGE-A1 epitopes.

## Materials and Methods

### *Cell lines, media, and reagents*

The EBV-transformed B (EBV-B)[3] cell lines and the melanoma cell lines were cultured in IMDM (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS (Life Technologies), 0.24 mM L-asparagine, 0.55 mM L-arginine, 1.5 mM L-glutamine (AAG), 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin. HeLa and COS-7 cells were maintained in DMEM (Life Technologies) supplemented with 10% FCS.

Human recombinant IL-2 was purchased from Eurocetus (Amsterdam, The Netherlands). The concentration needed to obtain half maximal proliferation of mouse CTLL-2 cells is 1 U/ml of IL-2. IL-7 was purchased from Genzyme (Cambridge, MA), and GM-CSF was from Schering-Plough (Brinny, Ireland). Human recombinant IL-4, IL-6, and IL-12 were produced in our laboratory. The concentration needed to obtain half maximal proliferation of mouse 7TD1 cells is 1 U/ml of IL-6 (23).

### *Recombinant viruses and infection of cell lines*

The recombinant canarypox ALVAC-MAGE-A1 (vCP 299), vaccinia WR-MAGE-A1 (vP 1188 and vP 1267), and the parental vaccinia (vP 1170) viruses were provided by Virogenetics (Troy, NY). The vaccinia encoding MAGE-A3 was provided by Vincenzo Cerundolo (Molecular Immunology Group, University of Oxford, Oxford, U.K.). Retroviral vector M1-CSM encodes the full length MAGE-A1 protein and the truncated form of the human low affinity nerve growth factor receptor ($\Delta$LNGFr). It was produced as previously reported (24). EBV-B cells or PHA-activated T cells were transduced by coculture with irradiated packaging cell lines producing the M1-CSM vector in the presence of polybrene (8 $\mu$g/ml). After 72 h, lymphocytes were harvested and seeded in fresh medium. The percentage of infected cells was evaluated 48 h later by flow cytometry for LNGFr expression with the mAb 20.4 (American Type Culture Collection, Manassas, VA). The LNGFr-positive cells were purified by magnetic cell sorting using rat anti-mouse IgG1-coated beads (Dynabeads M-450, Dynal, Oslo, Norway).

### *Dendritic cells and CD8$^+$ responder T cells*

Blood cells were collected as buffy-coat preparations from hemochromatosis patients LB1118, LB1801, and LB1137. PBMC were isolated by Lymphoprep (Nycomed, Oslo, Norway) density gradient centrifugation. To minimize contamination of PBMC with platelets, the preparation was first centrifuged at room temperature for 20 min at 160 g. After removal of the top 20–25 ml, which contained most of the platelets, the tubes were centrifuged at room temperature for 20 min at 350 g. To generate autologous dendritic cells, PBMC were depleted from T lymphocytes by rosetting with sheep erythrocytes (BioMerieux, Marcy l'Etoile, France) previously treated with 2-aminoethylisothiouronium (Sigma, St. Louis, MO). The lymphocyte-depleted PBMC were left to adhere for 1.5–2 h at 37°C in Falcon culture flasks (Becton Dickinson Labware, Meylan, France) at a density of 2–3 $\times$ 10$^6$ cells/ml in RPMI 1640 medium (Life Technologies) supplemented with AAG and 10% FCS (hereafter referred to as complete RPMI medium). Nonadherent cells were discarded, and adherent cells were cultured in the presence of IL-4 (10 ng/ml) and GM-CSF (100 ng/ml) in complete RPMI medium. Cultures were fed on days 2 and 4 by replacing one-half of the medium with fresh medium plus IL-4 (10 ng/ml) and GM-CSF (100 ng/ml). On day 5, the nonadherent cell population was used as a source of enriched dendritic cells.

Rosetted T cells were treated with $NH_4Cl$ (160 mM) to lyse the sheep erythrocytes and then washed. CD8$^+$ T lymphocytes were isolated from rosetted T cells by positive selection using an anti-CD8 mAb coupled to magnetic microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) and by sorting through a magnetic cell separation system, as recommended by the manufacturer. The lymphocytes were frozen and then thawed the day before the coculture with dendritic cells.

[3] Abbreviations used in this paper: EVB-B, EBV-transformed B; $\Delta$LNGFr, human low affinity nerve growth factor receptor; MOI, multiplicity of infection; AAG, 0.24 mM L-asparagine, 0.55 mM L-arginine, 1.5 mM L-glutamine.

### *Mixed lymphocyte-dendritic cell culture*

Dendritic cells (3 $\times$ 10$^6$) were infected with ALVAC-MAGE-A1 at a multiplicity of infection (MOI) of 30 in 1 ml of complete RPMI medium at 37°C under 5% $CO_2$. The infected dendritic cells were washed after 2 h. Autologous responder CD8$^+$ T lymphocytes (1.5 $\times$ 10$^5$) were mixed with infected dendritic cells (3 $\times$ 10$^4$) in microwells in 200 $\mu$l of complete IMDM in the presence of IL-6 (1000 U/ml) and IL-12 (10 ng/ml). On days 7 and 14, autologous dendritic cells that were either thawed or cultured for 5 days were infected with ALVAC-MAGE-A1 and used to restimulate the CD8$^+$ lymphocytes in medium supplemented with IL-2 (10 U/ml) and IL-7 (5 ng/ml). The responder CD8$^+$ T cells were assessed on day 21 or 28 for their capacity to lyse autologous EBV-B cells infected with vaccinia-MAGE-A1.

### *Cytotoxicity assay*

The cytotoxicity of an aliquot of each microculture was tested on autologous EBV-B cells infected with vaccinia-MAGE-A1 or control vaccinia. Infection was performed on 2 $\times$ 10$^6$ target cells for 2 h at an MOI of 20 in 500 $\mu$l of complete RPMI medium. Infected cells were washed, labeled with 100 $\mu$Ci of $Na(^{51}Cr)O_4$, and added to the responder cells at an E:T ratio of ~40:1. Unlabeled K562 cells were also added (5 $\times$ 10$^4$ per V-bottom microwell) to block NK activity. Individual microcultures were tested on each target in duplicate. Chromium release was measured after incubation at 37°C for 4 h.

### *Construction of a recombinant* Yersinia *and infection of EBV-B cell lines*

The wild-type *Yersinia enterocolitica* is an extracellular bacteria causing gastrointestinal syndromes in humans. *Y. enterocolitica* adhere to the surface of target cells and possess a virulence apparatus, called the Yop virulon, which enables the translocation of toxic effector proteins, including YopE, into the cytosol of the host cell (25, 26). A new strain, MRS40 (pABL403), has recently been constructed, where the genes encoding toxic Yop proteins are mutated or truncated (27). Interestingly, this polymutant strain maintains its ability to translocate proteins in fusion with a truncated YopE into the cytosol of eukaryotic cells, but it does not elicit cytotoxicity and can therefore be used as a vector to inject a protein into the cytosol of eukaryotic cells.

The sequence encoding protein MAGE-A1 was inserted in frame with a sequence encoding a truncated YopE, $YopE_{1-130}$, containing the first 130 aa of YopE. The open reading frame of *MAGE-A1* was amplified by PCR using a *MAGE-A1* cDNA cloned in pcDNAI/Amp (Invitrogen, Groningen, The Netherlands) as the template. The upstream primer, AAACTGCAGA TGTCTCTTGAGCAGAGGAGTC, consisted of the first nucleotides of the open reading frame of *MAGE-A1* preceded by a *Pst*I site. The downstream primer, AAACTGCAGTCAGACTCCCTCTTCCTCCTC, consisted of nucleotides complementary to the last nucleotides of the open reading frame of *MAGE-A1* followed by a *Pst*I site. The PCR product was digested with *Pst*I and inserted in frame with the truncated YopE at the *Pst*I site of vector pMS111 (25). pMS111-*MAGE-A1* was electroporated in *Escherichia coli* strain DH5$\alpha$F′IQ. The DNA of a recombinant clone was sequenced and then electroporated in *E. coli* strain SM10. Plasmid pMS111-*MAGE-A1* was mobilized by SM10 into *Y. enterocolitica* MRS40. Recombinant MRS40 were selected on agar-containing medium and supplemented with nalidixic acid (35 $\mu$g/ml), sodium m-arsenite (1 mM), and chloramphenicol (12 $\mu$g/ml) (28).

One colony of *Y. enterocolitica* MRS40 containing pMS111-*MAGE-A1* was then grown overnight at 28°C in Luria-Bertani medium supplemented with nalidixic acid (35 $\mu$g/ml), sodium m-arsenite (1 mM), and chloramphenicol (12 $\mu$g/ml). To obtain an optical density of 0.2 at 600 nm, this culture was diluted and cultured at 28°C for ~2 h. The bacteria were then washed in 0.9% NaCl and resuspended at 10$^8$/ml in 0.9% NaCl, assuming that a culture giving an $OD_{600}$ equal to 1 contains 5 $\times$ 10$^8$ bacteria/ml. Irradiated EBV-B cells were resuspended at 10$^6$ in 3.8 ml of RPMI without antibiotics, and supplemented with 10% FCS and AAG. Then, 200 $\mu$l of the bacterial suspension were added. Two hours after infection, gentamicin (30 $\mu$g/ml) was added for the next 2 h. The cells were finally washed three times before use as stimulator cells.

### *CD8$^+$ T cell clones*

The CD8$^+$ T cell microcultures that specifically recognized autologous EBV-B cells infected with vaccinia-MAGE-A1 construct were cloned by limiting dilution, using either autologous cells transduced with a retrovirus encoding MAGE-A1 or autologous EBV-B cells infected with *Yersinia*-MAGE-A1 as stimulator cells (5 $\times$ 10$^3$–10$^4$ cells/well in a 96-well plate). Allogeneic EBV-B cells (5 $\times$ 10$^3$–10$^4$ LG2-EBV cells/well in a 96-well

plate) were used as feeder cells. CTL clones were tested for lysis on autologous EBV-B cells infected with vaccinia-MAGE-A1. Established CTL clones were grown in complete IMDM supplemented with IL-2 (50 U/ml) and 0.5 $\mu$g/ml purified PHA and passaged with feeder cells ($1.5 \times 10^6$ LG2 EBV-B cells/well in a 24-well plate) at 1-wk intervals.

### *cDNA encoding HLA class I molecules*

The HLA-A3 coding sequence cloned in expression vector pcDNA3 (Invitrogen) was isolated from a cDNA library of cell line LB265-MEL. The HLA-A28 coding sequence was isolated from a cDNA library of cell line LB33-MEL cloned into expression vector pcDNA3 (29). The HLA-B53 coding sequence was amplified by RT-PCR using RNA of EBV-B cells of donor LB1801 as the template and cloned into pcDNA3. The HLA-Cw2 coding sequence was amplified by RT-PCR using RNA of EBV-B cells of donor LB1118 as the template and cloned into pcDNA3. The HLA-Cw3 coding sequence cloned in expression vector pCR3 was obtained from Dr. Schadendorf (Virchow Klinikum, Humboldt Universität zu Berlin, Berlin, Germany).

### *Transfection of HeLa cells or COS-7 cells and TNF assay*

HeLa ($2 \times 10^4$) or COS-7 ($1.5 \times 10^4$) cells distributed in flat-bottom microwells were cotransfected with 50 ng of pcDNAI/Amp containing the *MAGE-A1* cDNA and 50 ng of plasmid containing the coding sequences of each of the six putative HLA alleles using 1 $\mu$l of Lipofectamine (Life Technologies). Transfected cells were incubated for 24 h at 37°C and 8% $CO_2$. The transfectants were then tested for their ability to stimulate the production of TNF by the CTL clone. Briefly, 1500 CTL were added to the microwells containing the transfectants, in a total volume of 100 $\mu$l of complete IMDM supplemented with 25 U/ml of IL-2. After 24 h, the supernatant was collected and its TNF content was determined by testing its cytotoxic effect on WEHI-164 clone 13 cells in a MTT colorimetric assay (30–32).

### *Peptide recognition assay*

Peptides were synthesized on solid phase using F-moc for transient $NH_2$-terminal protection and were characterized using mass spectrometry. All peptides were >80% pure, as indicated by analytical HPLC. Lyophilized peptides were dissolved at 20 mg/ml in DMSO or at 2 mg/ml in DMSO/10 mM acetic acid and stored at −20°C. Target cells were labeled with $Na(^{51}Cr)O_4$, washed, and incubated for 15 min in the presence of peptide. CTL clone was then added at an E:T ratio of 5:1–10:1. Chromium release was measured after incubation at 37°C for 4 h.

## Results

For the purpose of identifying new Ags encoded by gene *MAGE-A1*, we stimulated in vitro lymphocytes, obtained from the blood of individuals without cancer, with autologous dendritic cells infected with an ALVAC virus engineered to contain the *MAGE-A1* sequence. The dendritic cells were obtained by culturing blood monocytes for 5–7 days in medium supplemented with GM-CSF and IL-4. Dendritic cells prepared by this procedure have been referred to as "immature dendritic cells" (33, 34).

### *Presentation of Ag by dendritic cells infected by ALVAC-MAGE*

As a preliminary experiment, we first examined the capacity of a recombinant ALVAC virus, encoding $\beta$-galactosidase, to infect dendritic cells. The cells were submitted to infection for 2 or 6 h, washed, cultured for 20 h, and tested for $\beta$-galactosidase activity. After 2 h of infection, the plateau of 25% of infected blue cells was reached at a MOI of 30, with <15% of mortality. We observed a much higher mortality after 6 h of infection without any increase in the percentage of infected cells.

We infected HLA-A1 dendritic cells at various MOI with ALVAC-MAGE-A1, an ALVAC vector containing the entire coding region of *MAGE-A1*. Then we examined the ability of these cells to present Ag to CTL. They proved capable of stimulating the release of IFN-$\gamma$ by CTL clone 82/30, which is directed against a MAGE-A1 epitope presented by HLA-A1 (Fig. 1) (35). In these experiments, the plateau of stimulation was also observed at an MOI of 30. Accordingly, this MOI was chosen to infect for 2 h the stimulator dendritic cells used in later experiments.

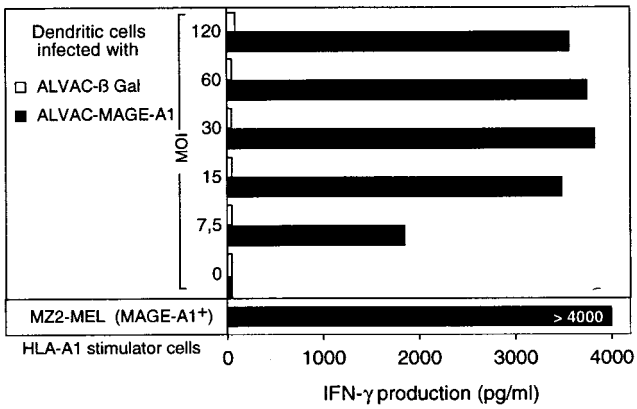


**FIGURE 1.** Dendritic cells infected with a canarypox virus, ALVAC, encoding *MAGE-A1* stimulate an anti-MAGE-A1 CTL clone. Dendritic cells of an HLA-A1 donor were infected with ALVAC-MAGE-A1 or ALVAC-$\beta$-galactosidase at various MOI for 2 h, washed, and distributed in microwells ($10^4$ cells/well). Three thousand cells of CTL clone 82/30, directed against a MAGE-A1 epitope presented by HLA-A1 (35), were then added. After 20 h, IFN-$\gamma$ produced by CTL 82/30 was measured by ELISA. The average results of triplicate cultures are shown here.

### *Stimulation of primary anti-MAGE CTL responses*

Dendritic cells were infected with ALVAC-MAGE-A1 and used to stimulate autologous $CD8^+$ T cells as summarized in Fig. 2. For each experiment, 96 microcultures were set up with $3 \times 10^4$ stimulator dendritic cells and $1.5 \times 10^5$ responder $CD8^+$ T cells in the presence of IL-6 and IL-12. Responder cells were restimulated once a week with autologous dendritic cells infected with ALVAC-MAGE-A1 in the presence of IL-2 and IL-7. Most of the microcultures multiplied by a factor ranging from 3 to 8. Responder cells were tested on day 21 and sometimes on day 28 for their lytic activity on autologous EBV-B cells infected with a vaccinia virus encoding MAGE-A1 (vaccinia-MAGE-A1). As a negative control, we used EBV-B cells infected with the parental vaccinia. The reason for using vaccinia instead of ALVAC was primarily that EBV-B cells infected with ALVAC are poor targets for chromium-release assays (data not shown). It was important to use as negative controls EBV-B cells infected with vaccinia instead of uninfected EBV-B cells because we observed that a significant number of microcultures have lytic activity directed against Ags shared by poxviruses ALVAC and vaccinia.

Four experiments were performed with blood cells of three individuals without cancer. In two experiments performed with cells of the same donor, the anti-MAGE-A1 reactivity was measured in 3 and in 2 microcultures out of 96. For the two other donors, the anti-MAGE-A1 reactivity was measured in 7 and 2 microcultures, respectively (Table I).

A number of microcultures displaying anti-MAGE-A1 reactivity were cloned by limiting dilution in the presence of autologous stimulator cells, IL-2, and allogeneic EBV-B cells as feeder cells (Fig. 2). To avoid the proliferation of anti-ALVAC CTL, we did not use cells infected with ALVAC-MAGE-A1 as stimulator cells. Instead, we used PHA-activated T cells transduced with a retrovirus encoding *MAGE-A1*, EBV-B cells transduced with the same retrovirus, or EBV-B cells infected with a *Yersinia enterocolitica* producing MAGE-A1 (*Yersinia*-MAGE-A1). These recombinant bacteria expressed a *yopE-MAGE-A1* fusion gene, allowing the translocation of a MAGE-A1 fusion protein into the cytosol of

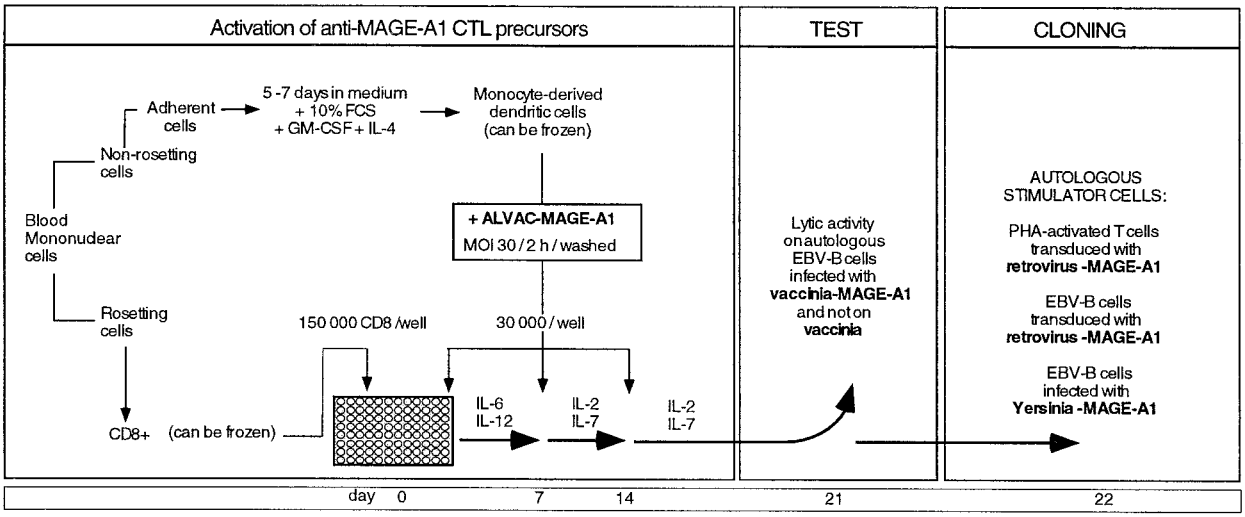


**FIGURE 2.** Overview of the procedure used to obtain anti-MAGE-A1 CTL clones

mammalian cells (see *Materials and Methods*). We verified that HLA-A1 EBV-B cells infected with *Yersinia*-MAGE-A1 were capable of stimulating the release of IFN-γ by CTL clone 82/30, which is directed against a MAGE-A1 epitope presented by HLA-A1 (data not shown). After a few weekly restimulations, the growing CTL clones were tested for their lytic activity on autologous EBV-B cells infected with vaccinia-MAGE-A1. Anti-MAGE-A1 CTL clones were obtained with each of the three types of stimulator cells. Afterwards, these clones were maintained in the presence of PHA, feeder cells, and cytokines, but without stimulator cells.

### *A MAGE-A1 peptide presented by HLA-A3*

From a microculture of lymphocytes of donor LB1118, who was typed HLA-A2, -A3, -B8, -B40, -Cw2, and -Cw7, clone LB1118-CTL 435/A9 (hereafter referred to as CTL clone 9) was obtained by limiting dilution using stimulator cells transduced with a retrovirus encoding MAGE-A1. CTL clone 9 recognized autologous EBV-B cells infected with vaccinia-MAGE-A1 (Fig. 3*A*) or transduced with a retrovirus encoding MAGE-A1 (data not shown).

To identify the HLA molecule that presents the MAGE-A1 peptide recognized by CTL clone 9, HeLa cells were transfected with a *MAGE-A1* cDNA, together with cDNAs coding for each of the putative HLA-presenting molecules, and these cells were used to stimulate the CTL clone. CTL clone 9 produced TNF upon stimulation by HeLa cells transfected with *MAGE-A1* and HLA-A3 (Fig. 3*B*).

To identify the MAGE-A1 peptide recognized by this CTL clone, we screened a set of peptides of 16 aa, which overlapped by 12 aa and covered the entire MAGE-A1 protein sequence. Autologous EBV-B cells were incubated with each of these peptides at

Table I. *Summary of the primary anti-MAGE-A1 CTL responses*

| Expt.[a] | Patient | HLA Subtype | Microcultures with Lytic Activity on Targets Infected with Parental Vaccinia[b] (out of 96) | Microcultures with Anti-MAGE-A1 Lytic Activity (out of 96) | Anti-MAGE-A1 CTL Clones Obtained | |
|---|---|---|---|---|---|---|
| | | | | | From microculture | Directed against an epitope presented by |
| 1 | LB1118 | A2, A3<br>B8, B40<br>Cw2, Cw7 | 14 | 3 | 1<br>2<br>3 | HLA-A3<br>No clone<br>No clone |
| 2 | LB1118 | A2, A3<br>B8, B40<br>Cw2, Cw7 | 19 | 2 | 1<br>2 | HLA-Cw2<br>Not yet defined[c] |
| 3 | LB1801 | A2, A28<br>B44, B53<br>Cw4, Cw5 | 3 | 7 | 1<br>2<br><br>3<br>4<br>5<br>6<br>7 | HLA-B53<br>HLA-A28<br>Not yet defined<br>HLA-A28<br>Not yet defined<br>Not yet defined<br>Not yet defined<br>No clone |
| 4 | LB1137 | A2, A3<br>B44, B60<br>Cw3, Cw5 | 17 | 2 | 1<br>2 | HLA-Cw3<br>No clone |

[a] $CD8^+$ lymphocytes were seeded in microwells and stimulated on days 0, 7, and 14 with dendritic cells infected with ALVAC-MAGE-A1. On day 21 or 28, individual microcultures were tested in duplicate for their lytic activity on autologous EBV-B cells infected with either parental vaccinia or vaccinia-MAGE-A1, at an E:T ratio of about 40:1. Unlabeled K562 cells were also added to block natural killer activity. Chromium release was measured after 4 h of incubation at 37°C.

[b] The lytic activity was higher than 20% both on autologous EBV-B cells infected with vaccinia or vaccinia-MAGE-A1. In the first experiment, 9 of 14 microcultures had no lytic activity against uninfected EBV-B cell targets, indicating that ALVAC and vaccinia vectors shared antigens recognized by CTL. In the other experiments this control target was not included.

[c] Anti-MAGE-A1 CTL clones were obtained but either proliferation was too low for further analysis or the MAGE-A1 epitope has not yet been identified fully.

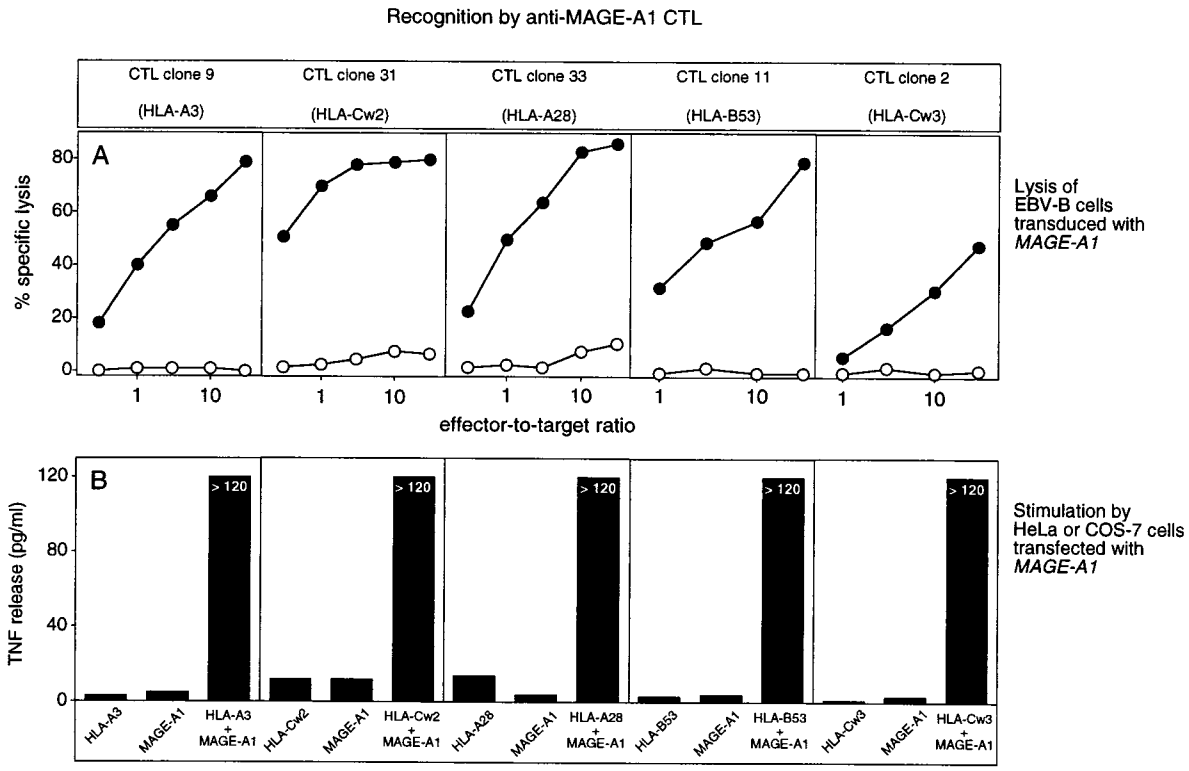


**FIGURE 3.** Recognition of *MAGE-A1*$^+$ cells by anti-MAGE-A1 CTL clones. *A*, EBV-B target cells were infected for 2 h with vaccinia-MAGE-A1 (●) at an MOI of 20, $^{51}$Cr-labeled, and incubated with the autologous CTL clone for 4 h. Targets infected with vaccinia (○) were used as a negative control. *B*, Recipient cells were HeLa cells for CTL 9 and COS-7 cells for the other clones. HeLa ($2 \times 10^4$) or COS-7 ($1.5 \times 10^4$) cells were cotransfected with sequences encoding MAGE-A1 and the HLA-presenting molecule using Lipofectamine. One day after transfection, 1500 CTL were added into microwells containing the transfected cells. TNF production was estimated after overnight coculture by testing the toxicity of the supernatants for the TNF-sensitive WEHI-164 clone 13 cells.

a concentration of 1 $\mu$M, and these cells were tested for recognition by CTL clone 9 in a chromium release assay. Peptide STSCILESLFRAVITK scored positive. Its sequence was then screened for the presence of a shorter peptide with consensus anchor residues for HLA-A3: a L, V, or M in position 2, and a K, Y, or F in position 9 (36). Only nonapeptide SLFRAVITK (MAGE-A1$_{96-104}$) fulfilled these conditions. It was tested in a cytotoxicity assay with CTL clone 9 and produced half maximal lysis of autologous EBV-B target cells at ~2 nM (Fig. 4*A*).

### *A MAGE-A1 peptide presented by HLA-Cw2*

From another microculture of lymphocytes of donor LB1118, clone LB1118-CTL 466/D3.31 (hereafter referred to as CTL clone

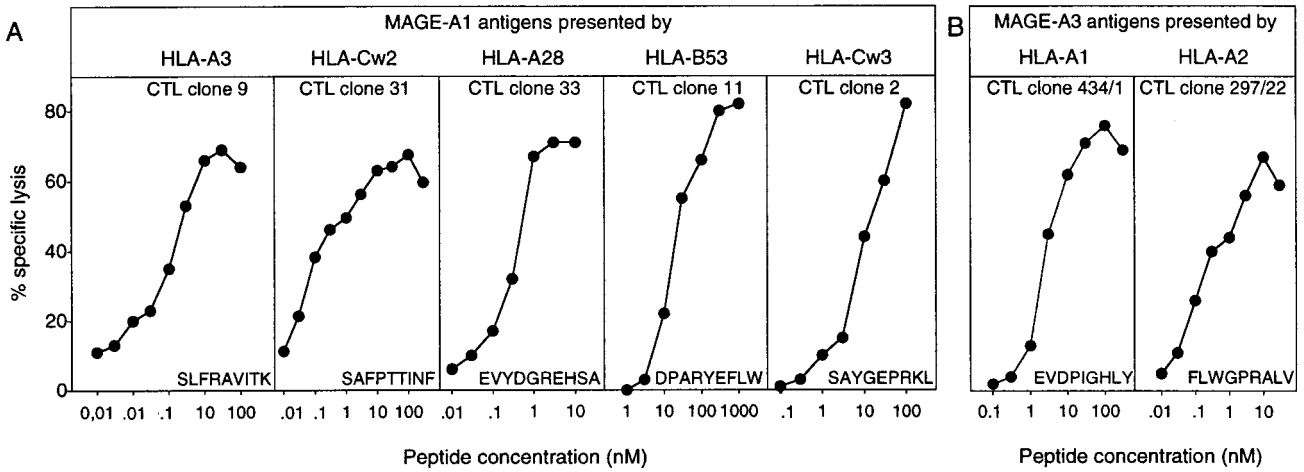


**FIGURE 4.** Lysis by anti-MAGE CTL clones of autologous EBV-B cells incubated with peptide. *A*, Lysis by anti-MAGE-A1 CTL clones. Targets were $^{51}$Cr-labeled and incubated for 4 h with the CTL, at an E:T ratio of 5:1 for CTL clone 9 and of 10:1 for the other clones, in the presence of the peptide at the concentrations indicated. Chromium release was measured after 4 h. *B*, Lysis by anti-MAGE-A3 CTL clones. CTL 297/22 recognizes a MAGE-A3 epitope presented by HLA-A2 (15). CTL 434/1 recognizes a MAGE-A3 epitope presented by HLA-A1 (40). They were used at an E:T ratio of 10:1.

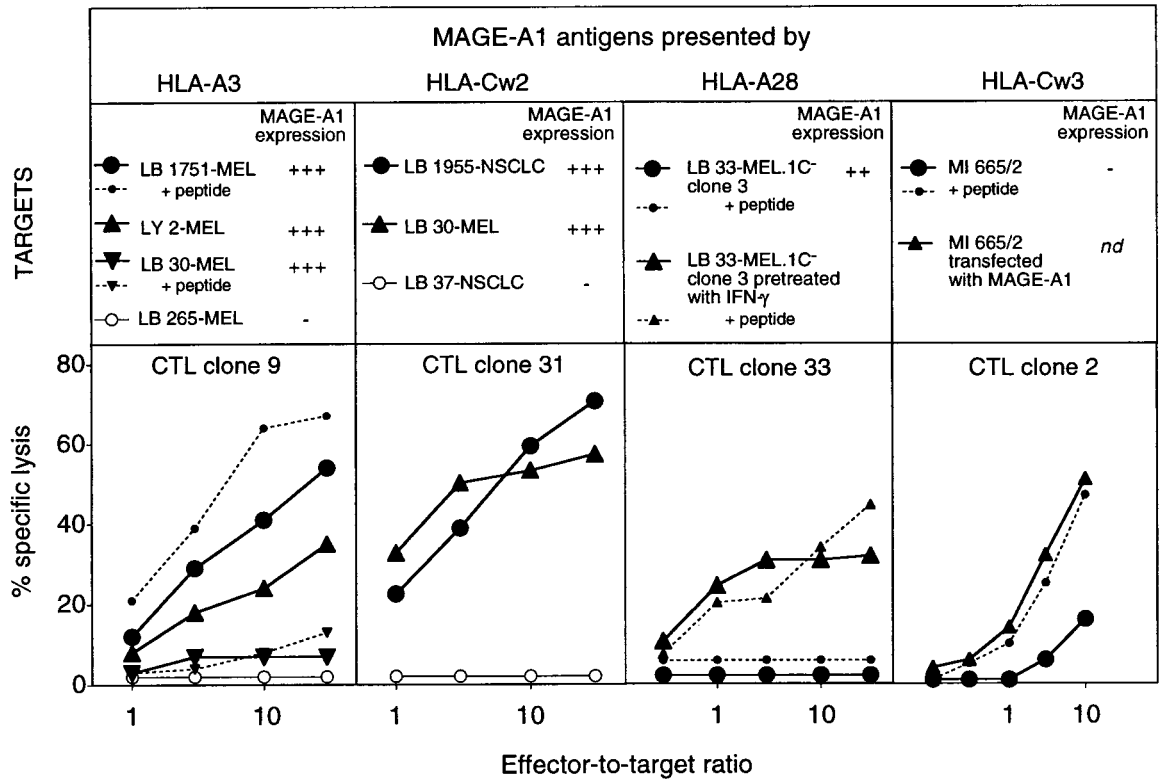


**FIGURE 5.** Lysis of tumor cell lines by anti-MAGE CTL clones. Tumor cell lines were melanoma cell lines (MEL) or non-small cell lung cancer cell lines (NSCLC). Target cells were $^{51}$Cr-labeled and incubated with CTL at various E:T ratios. Chromium release was measured after 4 h. Expression of *MAGE-A1* was determined by RT-PCR analysis and scored according to the band intensity of the PCR products. Control experiments conducted with serial dilutions of the RNA of melanoma line MZ2-MEL-3.0, which expresses *MAGE-A1*, indicated that the levels of expression reported here are approximately: +++, 200–50% of the expression level of this reference cell line; ++, 50–10%; +, 10–1%; −, <1% (no product observed).

31) was also obtained after cloning with stimulator cells transduced with a retrovirus encoding MAGE-A1. It lysed autologous EBV-B cells infected with vaccinia-MAGE-A1 (Fig. 3*A*) or transduced with a retrovirus encoding MAGE-A1 (data not shown). CTL clone 31 produced TNF upon stimulation by COS-7 cells transfected with HLA-Cw2 and *MAGE-A1* (Fig. 3*B*).

The screening of the set of peptides of 16 aa with CTL clone 31 was negative. We tested another set of MAGE-A1 peptides of 12 aa that overlapped by 8 aa. Peptide ASAFPTTINFTR scored positive in contrast to peptide SPQGASAFPTTINFTR, which scored negative. In the absence of information relative to the anchor residues binding to HLA-Cw2 molecules, many shorter peptides were tested. Nonapeptide SAFPTTINF (MAGE-A1$_{62–70}$) was found to be the shortest peptide capable of efficiently sensitizing autologous target cells to lysis by CTL clone 31, with a half maximal lysis obtained at ~0.1 nM (Fig. 4*A*).

### *A MAGE-A1 peptide presented by HLA-A28*

From a microculture set up with lymphocytes of donor LB1801, who was typed HLA-A2, -A28, -B44, -B53, -Cw4, and -Cw5, clone LB1801-CTL 456/H8.33 (hereafter referred to as CTL clone 33) was obtained using EBV-B cells infected with *Yersinia*-MAGE-A1 as stimulator cells. CTL clone 33 lysed autologous EBV-B cells infected with vaccinia-MAGE-A1 (Fig. 3*A*). It produced TNF upon stimulation by COS-7 cells transfected with HLA-A28 and *MAGE-A1* (Fig. 3*B*).

When the two sets of MAGE-A1 overlapping peptides were tested for recognition by CTL clone 33, peptides MEVYDGREH SAYGEPR and MEVYDGREHSAY scored positive. No shorter peptide contained the two consensus anchor residues for HLA-A*6801, which is a subtype of HLA-A28: a V or T in position 2 and a R or K in position 9 (36). Shorter peptides with only a V in position 2 were tested. Decapeptide EVYDGREHSA (MAGE-A1$_{222–231}$) was found to be the shortest peptide capable of efficiently sensitizing autologous target cells to lysis by CTL clone 33, with a half maximal lysis of target cells at ~0.3 nM (Fig. 4*A*).

### *A MAGE-A1 peptide presented by HLA-B53*

From a second microculture set up with cells of donor LB1801, clone LB1801-CTL 456/H7.11 (hereafter referred to as CTL clone 11) was obtained using EBV-B cells infected with *Yersinia*-MAGE-A1 as stimulator cells. It lysed autologous EBV-B cells infected with vaccinia-MAGE-A1 (Fig. 3*A*).

When the set of peptides was tested for recognition by CTL clone 11, peptides QVPDSDPARYEFLWGP and SDPARYEFL WGPRALA scored positive. By testing as target cells a pool of allogeneic EBV-B cell lines pulsed with these peptides, we came to the tentative conclusion that B53 was the presenting molecule. The *HLA-B53* gene of donor LB1801 was cloned and transfected into COS-7 cells together with a *MAGE-A1* cDNA. The transfected cells stimulated CTL clone 11 to produce TNF (Fig. 3*B*). The sequences of the two long peptides were screened for the presence of a shorter peptide with consensus anchor residues for HLA-B53, namely a P in position 2, and a F, I, L, M, V, W, or Y in position 9 (36). Only nonapeptide DPARYEFLW (MAGE-A1$_{258–266}$) fulfilled these conditions. It produced half maximal lysis of autologous EBV-B target cells at ~20 nM (Fig. 4*A*).

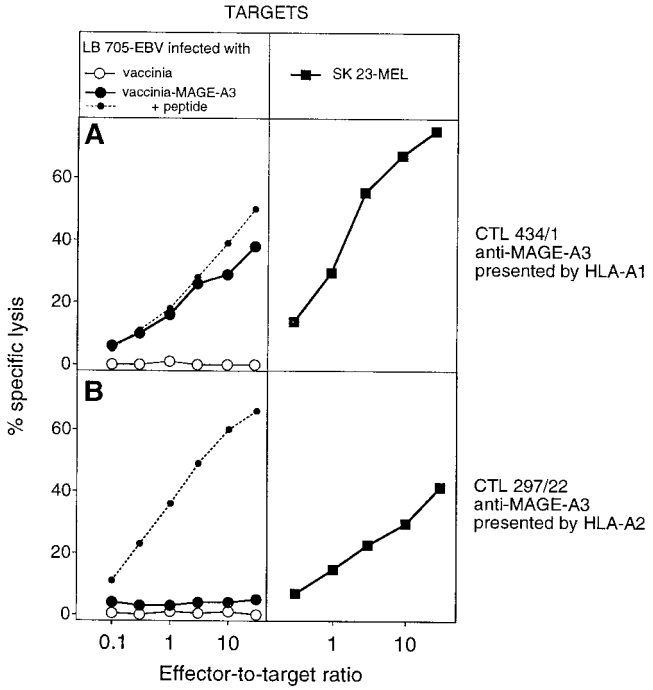


**FIGURE 6.** Lysis of vaccinia-infected EBV-B cells and of a tumor cell line by anti-MAGE-A3 CTL clones. LB705 EBV-B target cells were infected for 2 h with vaccinia-MAGE-A3 (●) at an MOI of 20, $^{51}$Cr-labeled, and incubated with autologous CTL clones 434/1 (*A*) or 297/22 (*B*) for 4 h. Targets infected with vaccinia (○) were used as a negative control. Dotted lines represent the lysis on targets cells incubated (*A*) with peptide EVDPIGHLY at a final concentration of 1 $\mu$M or with peptide FLWGPRALV at a final concentration of 500 nM (*B*). HLA-A1 and A2 SK 23-MEL tumor cells were $^{51}$Cr-labeled and incubated with CTL at various E:T ratios. Chromium release was measured after 4 h.

*A MAGE-A1 peptide presented by HLA-Cw3*

From a microculture set up with lymphocytes from donor LB1137, who was typed HLA-A2, -A3, -B44, -B60, -Cw3, and -Cw5, we obtained clone LB1137-CTL 462/F3.2 (hereafter referred to as CTL clone 2) by limiting dilution using EBV-B cells infected with *Yersinia*-MAGE-A1 as stimulators. This clone lysed autologous EBV-B cells infected with vaccinia-MAGE-A1 (Fig. 3*A*). CTL clone 2 produced TNF upon stimulation by COS-7 cells transfected with HLA-Cw3 and *MAGE-A1* (Fig. 3*B*).

When the set of peptides was tested for recognition by CTL clone 2, DGREHSAYGEPRKLLT scored positive. Nonapeptide SAYGEPRKL (MAGE-A1$_{230-238}$), which had a terminal residue corresponding to the F, M, L, I consensus for HLA-Cw3 (36), produced half maximal lysis of targets cells at ~10 nM (Fig. 4*A*). Remarkably, a CTL clone, obtained in a mixed lymphocyte-tumor cell culture set up with cells from a melanoma patient, had been previously shown to recognize the same peptide presented by HLA-Cw16 (37).

*Lysis of tumor cell lines by the anti-MAGE-A1 CTL clones*

In so far as we had at our disposal tumor cell lines that expressed *MAGE-A1* and that were derived from patients carrying appropriate HLA alleles, we tested their sensitivity to lysis by the anti-MAGE-A1 CTL clones. Such tumor cell lines were found for the CTL clones that recognized MAGE-A1 epitopes presented by HLA-A3, -Cw2, and -A28. Some of these tumor cell lines were lysed very well (Fig. 5). Others were poorly lysed or not lysed at all, even though their level of expression of *MAGE-A1* appeared to be appropriate. As it is well known that many tumors down-regulate or loose HLA expression (38, 39), we examined whether this could account for negative results. HLA-A3 and -A28 tumor cells that were not lysed by the anti-MAGE-A1 CTL clones were incubated with the relevant peptides. The peptide-pulsed cells were also insensitive to the CTL, confirming the HLA defect. In line with this interpretation, IFN-$\gamma$ treatment, which is known to up-regulate HLA expression in some cells, increased the lysis of an HLA-A28 tumor cell line, both in the presence and in the absence of peptide. No HLA-Cw3 cell line expressing *MAGE-A1* was found but a HLA-Cw3 cell line was transfected with *MAGE-A1* and was found to be efficiently lysed. No HLA-B53 cell line was available.

Because our present approach is based on stimulation with cells that have been transduced with the *MAGE-A1* gene, it ought to lead to epitopes that are well processed. This finding is in contrast with a previous approach, that involved stimulation with cells pulsed with a synthetic peptide (13–18). This previous approach produced CTL clones that recognized peptides that were poorly or not processed, as well as peptides that were well processed. For instance, CTL clone 434/1 was isolated by the peptide approach. It recognized a well-processed MAGE-A3 epitope presented by HLA-A1 (Figs. 4*B* and 6*A*). This epitope had been identified previously using a CTL clone obtained by autologous mixed lymphocyte-tumor cell culture (40). As shown in Fig. 6*A*, CTL 434/1 clearly recognized autologous EBV-B cells infected with vaccinia-MAGE-A3 and an HLA-A1 tumor cell line expressing *MAGE-A3*. In contrast, a MAGE-A3 epitope presented by HLA-A2, which was also identified by the peptide approach (15), appeared to be poorly processed: EBV-B cells infected with vaccinia-MAGE-A3 were not lysed at all by the CTL clone 297/22, even though they were recognized very well after pulsing with the peptide (Figs. 4*B* and 6*B*). Nevertheless, some tumors that appeared to express a high level of MAGE-A3 showed significant lysis by CTL clone 297/22 (Fig. 6*B*) (15). Taken together, our results show that when a CTL clone efficiently lyses EBV-B cells transduced with the gene coding for the relevant Ag, it also lyses tumor cell lines that express the relevant gene and HLA molecule.

## Discussion

The procedure described here appears to be efficient for the activation of anti-MAGE CTL precursors, resulting in the isolation of permanent anti-MAGE CTL clones. It is worth noting that our approach does not require blood cells from cancer patients. Among the CD8$^+$ T lymphocytes of normal donors, anti-MAGE CTL were obtained at a frequency of $\sim 2 \times 10^{-7}$. The frequency of CTL precursors that was observed previously with the peptide stimulation approach was $\sim 6 \times 10^{-7}$ for the MAGE-A3 peptide presented by HLA-A2 and $\sim 2 \times 10^{-7}$ for the MAGE-A3 peptide presented by HLA-A1 (41). Because several peptides might be presented by one of the six HLA class I molecules, a higher frequency of anti-MAGE CTL might have been expected with our approach. The low frequency that we observed may be due to a lower number of antigenic complexes on infected dendritic cells, compared with peptide-pulsed cells, resulting in the activation of only those CTL that had high affinity receptors.

A drawback of our approach, which is based on the use of a recombinant virus to obtain dendritic cells that present the Ag, is that CTL precursors directed against viral epitopes are activated. We circumvented this problem by using different vectors for the stimulation of the microcultures, for the lytic assay with the responder T cells, and for the cloning step. The cloning was successfully achieved using autologous stimulator cells transduced

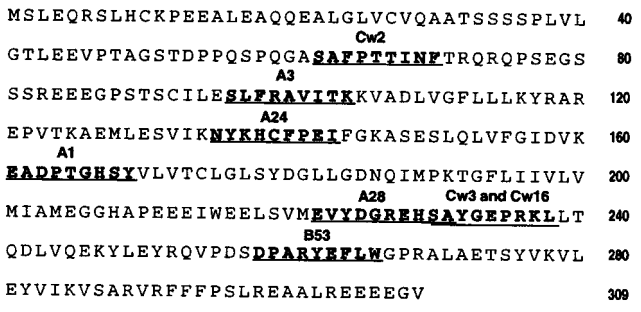


**FIGURE 7.** Sequence of the MAGE-A1 protein and position of the MAGE-A1 CTL epitopes. The sequences corresponding to the peptides recognized by a CTL clone on HLA-A3, -A28, -B53, -Cw2, -Cw3 (this manuscript), or on HLA-A1 (35), HLA-A24 (18), HLA-Cw16 (37) are underlined.

with a retrovirus encoding MAGE-A1 or infected with a recombinant *Yersinia*-MAGE-A1. It appears that a recombinant adenovirus is as suitable as ALVAC for the production of dendritic cells presenting MAGE Ags. In a search for Ags encoded by *MAGE-A4*, CTL were obtained upon primary stimulation of CD8 T cell populations with autologous dendritic cells infected with a recombinant adenovirus containing the MAGE-A4 coding sequence (M. T. Duffour and P. van der Bruggen, manuscript in preparation).

Our approach is not restricted to peptides selected a priori on the basis of consensus anchor residues and high affinity for HLA molecules measured in vitro. This is advantageous as CTL clones have occasionally been found to recognize peptides that do not contain consensus anchor residues, like the peptide presented by HLA-A28 reported here, or that do not have high affinity in vitro for the presenting molecule (42, 43). This procedure could also lead to the identification of antigenic peptides that result from posttranslational modifications (44, 45). These peptides would be missed by the peptide stimulation approach. Moreover, after infection with viral vectors containing an entire *MAGE* coding sequence, only the antigenic peptides processed efficiently by the infected dendritic cells are able to activate anti-MAGE CTL. These peptides are most likely to be those that are also well processed and presented at the surface of $MAGE^+$ tumor cells.

In a recently completed clinical trial, metastatic melanoma patients with measurable disease received, at monthly intervals, three s.c. injections of a MAGE-A3 peptide presented by HLA-A1 (46, 47). Significant tumor regressions were observed for 7 patients out of the 25 who received the complete treatment. Three regressions were complete and two of these led to a disease-free state that persisted for more than 2 years after the beginning of treatment. In another clinical trial, four patients with advanced melanoma were immunized with autologous dendritic cells pulsed with two HLA-A1-binding peptides derived from MAGE-A3 and MAGE-A1 (48). A partial response was reported for one patient. Immunization of two other patients with dendritic cells pulsed with HLA-A2-binding melanoma differentiation peptides in addition to the MAGE peptides did not result in tumor regression. If therapeutic vaccination with synthetic peptides or with APCs loaded with synthetic peptides proves to be effective, the identification of additional *MAGE*-encoded tumor Ags will be needed to cope with HLA restriction and to permit concurrent immunization against several Ags.

Three MAGE-A1 epitopes have been found previously to be presented by HLA-A1, -A24, or -Cw16 (Fig. 7), which are expressed by 26, 20, and 7% of Caucasians, respectively (18, 35, 37, 49). The five new MAGE-A1 epitopes are presented by HLA-A3, -A28, -B53, -Cw2, or -Cw3 molecules (Fig. 7), which are expressed by 25, 9, 1, 8, and 24% of Caucasians, respectively (49). Taken together with the three first peptides, MAGE-A1 epitopes are now available for 79% of Caucasians. We are fully aware that each epitope described here was identified with CTL isolated from only one individual and that the immunogenicity of these peptides may vary in different individuals.

Immunization by injection of purified proteins or recombinant viruses harboring large MAGE sequences, or by reinfusion into patients of autologous APCs infected with these viruses represent other therapeutic possibilities. One criterion of eligibility in a clinical trial recently initiated with a MAGE-A3 recombinant protein was the HLA type: the patients had to express either HLA-A1, A2, or B44 molecules, which have previously been shown to present a MAGE-A3 peptide to CTL (15, 16, 40). The results presented here for MAGE-A1 suggest that many if not all HLA molecules are able to present a MAGE-A1 peptide to CTL. Therefore, we feel that in future clinical trials using MAGE-A1 protein or viruses encoding MAGE-A1, it will possible to disregard the HLA type of the patients.

In future trials, it will be essential to have reliable monitoring of the CTL response against the immunizing Ag. One promising possibility, which nevertheless restricts the analysis to certain epitopes, is the use of a set of relevant peptides in combination with soluble HLA tetramers to label TCRs directly (50, 51). Only such a detailed analysis of the anti-MAGE-A1 CTL responses of patients will provide information on the immunogenicity of the various MAGE-A1 epitopes. The identification of a number of antigenic peptides, such as the ones described here, and the availability of permanent CTL clones directed against these peptides will be essential for this approach.

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