ORIGINAL ARTICLE

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Induction of cytolytic T lymphocytes by immunization of mice with an adenovirus containing a mouse homolog of the human MAGE-A genes

Abstract The genes of the *MAGE-A* family code for antigens that are strictly tumor-specific and are shared by many human tumors. Melanoma patients have been immunized against these antigens and some tumor regressions have been observed. However, no unequivocal evidence of cytolytic T cell responses has been obtained by analyzing the blood lymphocytes of these patients. Hence it was considered worthwhile to examine in mouse systems whether or not immunization against antigens derived from the mouse Mage homologs can produce cytolytic T cell responses. We have identified an antigenic peptide encoded by mouse gene *Mage-a2*, and here we show that immunization of DBA/2 mice with a recombinant adenovirus containing either just the sequence encoding this peptide or a large part of the *Mage-a2* coding sequence produces strong cytolytic T cell responses. The Mage-a2 system should prove useful for the comparison of vaccination modalities that could be applied to human patients in therapeutic vaccination trials with MAGE antigens.

Key words Cytotoxic T lymphocytes · Mouse · Antigen · Adenovirus · Tumor immunity

Introduction

Human tumors carry antigens recognized by autologous cytolytic T lymphocytes (CTL). Some of these antigens are encoded by genes of the *MAGE* family, which are not expressed in normal cells except for male germ-line cells, which do not express HLA molecules on their surface and therefore cannot present antigens to T cells. These genes are expressed in an important fraction of tumors of various histological types and their expression in tumors is linked with the demethylation of their promoter regions [9, 11, 46]. Three clusters of *MAGE* genes were identified on the X chromosome. The *MAGE-A* cluster is located in Xq28 and comprises 12 genes [9, 34]. The six genes of the *MAGE-B* cluster are located in Xp21.3 and Xp22 [8, 22, 24, 31]. Three genes constituting the *MAGE-C* cluster located in Xq26–27 have recently been identified [22, 23]. Other genes expressed only in tumors and in male-germ cells, have been identified, notably the *BAGE*, *GAGE* and *LAGE-1/ NY-ESO-1* families [2, 7, 20, 44].

A clinical trial involving immunization with an antigenic peptide encoded by *MAGE-A3* and presented by HLA-A1 has recently been completed [25]. Out of 25 melanoma patients with measurable tumors who received the full schedule of three monthly injections, seven showed tumor regressions, including three complete regressions. In other trials involving immunization with dendritic cells pulsed with MAGE-A1 and MAGE-A3 peptides presented by HLA-A1 or HLA-A2, partial tumor regressions were observed in patients who were treated [32, 39].

Several possible causes could explain why the majority of patients fail to respond to this therapy. Besides the loss of expression of tumor antigen, the lack of HLA molecule or a profound immunosuppression state of the patient, an important reason may be the lack of efficacy of the vaccine. We have therefore devoted considerable effort to comparing various modes of immunization in mouse tumor systems. We have identified, on mastocytoma P815, antigen P815AB that is encoded by *P1A*, a MAGE-like gene which is expressed only in tumors and in male germ-line cells [21, 41, 43]. We found some effective ways to induce a CTL response against antigen P815AB: immunizations with peripheral blood lympho-

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cytes or dendritic cells pulsed with peptide and combined with IL-12 [16]; recombinant adenoviruses containing a minigene coding for the antigenic peptide [50]; and, to a lesser extent, peptide combined with an adjuvant containing QS21 and MPL (SBAS-1c) and IL-12 [36].

We sought to confirm these results with a mouse tumor antigen that would be the best possible equivalent of MAGE antigens in humans. Recently, a family of eight *MAGE* homologs was characterized in the mouse and located to chromosome X [10]. The genes were called *Mage-a*, as their coding sequences displayed a slightly higher degree of nucleotide identity with the human *MAGE-A* than with the human *MAGE-B* or *MAGE-C* coding sequences. Gene *Mage-a2* contains three short exons in front of a fourth exon that comprises a long open reading frame coding for a protein of 320 amino acids. This gene was shown by sequencing of RT-PCR products to be expressed in mouse melanoma cell line B78H1. It is not expressed in normal adult tissues except in male germ-line cells [10].

We report here the identification of an antigenic peptide encoded by *Mage-a2*, and we show that recombinant adenoviruses containing *Mage-a2* sequences are effective in generating CTL responses in vivo.

Materials and methods

Mice and immunization

DBA/2 female mice were derived from an inbred colony (Iffa Credo, Lyon, France) and raised in specific pathogen-free conditions in our animal facilities. The mice were 12 weeks old. For the immunization with peptide, they received in the two footpads 50 µl of an emulsion containing a 1:1 mixture of 25 µg purified peptide in PBS and adjuvant DQS21-MPL (SBAS-1c, prepared by Smith-Kline-Beecham Biologicals, Rixensart, Belgium). Four injections were performed at 2-week intervals. For the immunizations with recombinant adenoviruses, the mice were injected once with a total of 10^9 plaque-forming units (pfu) injected either intradermally (i.d.) into each ear in 50 µl phosphate-buffered serum (PBS) or intraperitoneally (i.p.) in 100 µl PBS. All animals in the study were kept according to the Belgian legislation and the local ethics committee approved the animal studies.

Cell lines

Mastocytoma P815 was obtained in a DBA/2 mouse treated with methylcholanthrene. Antigen-loss variant P1.ist A^-B^-, called P1.204 and designated $P1.AB^-$ in this manuscript, is a subclone obtained from a mouse injected with the P815 clonal cell line. From clone P1.204, antigen-loss variant $P1.A^-B^-C^-D^-$, hereafter called $P1.ABCD^-$, was obtained after successive in vitro selections with CTL clones directed against antigens P815C [42], and -D [4]. P1.HTR is a highly transfectable recipient cell line derived from P815 [47]. This cell line does not express the *Mage-a2* gene. The P1.HTR.B7 was obtained by co-transfection of gene B7.1 cloned in vector pCDSRα and pSVtkneoβ [15, 33]. The B78H1 is a mouse melanoma cell line expressing low levels of class I H-2^b molecules. The EL4 6.1.10 is a clone derived from EL4 lymphoma that produces IL-2 upon phorbol myristate acetate (PMA) induction at 10 ng/ml for 24 h [17]. These cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). Human 293-EBNA cells (Invitrogen, Carlsbad, Calif.) were maintained in the same medium. The WEHI-164 clone

13 cells [14] were cultured in RPMI 1640 with 5% FCS. The RMA-S.K^d (a gift from F. Fallarino, Dept. Experimental Medicine, Perugia, Italy) is the TAP2-deficient RMA-S cell line expressing low levels of H-2^b class I molecules that was cotransfected with the K^d gene and pSVtkneoβ. These cells were cultured in the previous medium supplemented with L-arginine.HCl (116 mg/l), L-asparagine (36 mg/l), L-glutamine (216 mg/l), glucose (4.5 g/l), 10 mM Hepes and 5×10^{-5} M 2-mercaptoethanol (hereafter called MLC medium). Cells were incubated at 37 °C in a 8% CO_2 atmosphere.

Mixed lymphocyte-tumor cell culture

Spleen cells (5×10^6) from mice injected 2 weeks before with peptide and adjuvant were stimulated with either 5×10^6 irradiated (3,000 rad) syngeneic splenocytes pulsed with peptide, or 2×10^5 irradiated (10,000 rad) P1.204 cells pulsed with peptide. For the pulse, the cells were incubated with 10 µM peptide for 2 h in medium containing 2% FCS and then washed with mixed lymphocyte culture (MLC) medium. The splenocytes and stimulating cells were cultured in 2 ml MLC medium. After 7 days, the responder cells were tested in a chromium release assay. Similarly, the splenocytes from mice injected 2 weeks before with recombinant adenoviruses, were stimulated with 2×10^5 irradiated cells, either P1.ABCD$^-$ cells pulsed with 10 µM peptide or transfected cells producing a truncated Mage-a2 protein [amino acids (aa) 1–200].

CTL clones

All the CTL clones were obtained by limiting dilution. Limiting numbers of the stimulated lymphocytes were seeded with 10^6 irradiated syngeneic spleen cells and 2×10^4 irradiated P1.ABCD$^-$ cells pulsed with 10 µM peptide or cells transfected with the truncated *Mage-a2* gene, in 200 µl MLC medium containing 50% supernatant of a secondary MLC, as a source of cytokines [26]. The microcultures were restimulated under the same conditions at day 7 and day 14. After 3 weeks, the proliferating clones were tested in a chromium release assay against cells pulsed with the Mage-a2 peptide. The lytic clones were transferred to wells containing 5×10^6 irradiated spleen cells and 10^5 irradiated cells pulsed with peptide or transfectants expressing the truncated Mage-a2 protein, in 2 ml MLC medium containing 50% supernatant of secondary MLC. Using these conditions, CTL (10^5/well) were cultured every week. Subsequently, the secondary MLC supernatant was replaced by 0.5% supernatant of EL4 6.1.10 cells stimulated with PMA (10 ng/ml) for 24 h.

Chromium and tumor necrosis factor release assays

The chromium release assay has been described [3]. Briefly, MLTC responder cells or CTL clones are mixed at various ratios with 2000 ^{51}Cr-labeled target cells. After 4 h, the radioactivity released in the supernatant is measured. Some results of lytic activity are expressed in lytic units (LU) defined as the number of spleen cells that lyse 50% of 10^4 target cells in 4 h. This number is estimated from the specific release obtained at various effector-to-target ratios by means of regression $(1 - e^{-kx})$ [5].

For the tumor necrosis factor (TNF) release assay, 2000 CTL cells were incubated with 2×10^4 tumor cells. After 24 h, the supernatant was collected and its TNF content was measured by testing its cytotoxic effect on WEHI-164 clone 13 cells in an MTT colorimetric assay [18, 40].

Construction of two pAdeno.SRαMage-a2 recombinant plasmids

For the construction of a recombinant adenovirus encoding a truncated Mage-a2 protein, a PCR fragment encoding the first 200 amino acids of this protein was obtained with the following primers: sense 5′-GGAATTCCATAATG.GCT.GAC.TCC.CA-T.AAC.AC-3′ and anti-sense 5′-GGAATT.CTA.TCC.CTT.-CAT.GAA.AAT.GAC.ACT.CAG-3′. This fragment was cloned

into the *Eco*RI site of pCDSRα (a gift from K. Moore, DNAX Institute, Palo Alto, Calif.) and the sequence was verified. The *Mage-a2* coding sequence and the SRα promoter were then removed and cloned into plasmid pCO1 (a gift from C. Orsini, Rhône-Poulenc Rorer Gencell, Vitry-sur-Seine, France) to generate plasmid pAdeno.SRαMage-a2(1–200).

pAdeno.SRαM$_{172–180}$ was similarly prepared. Briefly, oligonucleotides were synthesized that encompassed the sequence encoding the M$_{172}$ peptide (TYDGMMTDV), with the addition of an ATG upstream, a STOP codon downstream and *Eco*RI restriction sites at both ends of the minigene. The minigene was cloned into the *Eco*RI site of plasmid pCDSRα and the sequence was verified. pAdeno.SRαM$_{172–180}$ was similarly prepared by cloning into plasmid pCO1.

Construction and propagation of the recombinant adenoviruses

The Adeno.Mage-a2 (1–200) and Adeno.M$_{172–180}$ recombinant adenoviruses were produced in cell line 293 by homologous recombination between one of the two recombinant plasmids and Cla I-restricted Adeno.RSVβgal genomic DNA, [38]. The recombinant adenoviruses were propagated in 293 cells, purified by double cesium chloride density gradient centrifugation and extensively dialyzed. The viral stocks were stored in aliquots with 10% glycerol at −80 °C and titrated by plaque assay on 293 cells, as described [50].

Transfection of the K^d molecule into melanoma B78H1

Transfection using the DNA/calcium phosphate coprecipitation method was previously described [52]. Briefly, B78H1 were seeded in bottles at 5×10^5 cells. The next day, cells were cotransfected with 20 μg pcDNAI-K^d (a gift from J. Bilsborough, Ludwig Institute, Brussels) and 2 μg pSVtkneoβ. The cells growing in 1.5 mg/ml of G418 were cloned by limiting dilution, in microplates at 0.3 or 0.1 cell/well in 100 μl medium. The expression of the K^d molecule was analyzed by FACS (FACScan, Becton Dickinson, San Jose, Calif.) using mAb SF1 1.1.1 (hybridoma cell culture supernatant: ATCC HB-159) and GAM-FITC (Becton Dickinson). To improve the level of class I expression, the cells were preincubated in 25% of secondary MLC supernatant as a source of IFNγ for 24 h.

Results

Selection of a Mage-a2 peptide binding to *H*-2K^d

We set out to identify Mage-a2 antigenic peptides recognized by T lymphocytes on the *H*-2K^d molecule. The Mage-a2 putative protein sequence was searched for peptides with consensus anchor residues for binding to *H*-2K^d, that is with a tyrosine in position 2 and a valine, leucine or isoleucine in position 9. We found three sequences with such anchor residues. The three peptides were synthesized and their binding was assessed on *H*-2^b deficient RMA-S cells transfected with a *H*-2K^d gene. Only peptide Mage–a2$_{172–180}$ (M$_{172}$: TYDGMMTDV) (Fig. 1) showed binding to K^d, as seen by its ability to increase the level of K^d molecules on the surface of RMA-S cells (Fig. 2).

In vivo priming of CTL by the Mage-a2/K^d peptide

To test the immunogenicity of the M$_{172}$ peptide, DBA/2 mice were injected four times with this peptide mixed with the SBAS-1c adjuvant. In a first experiment, spleen cells from immunized animals were stimulated with syngeneic P1 AB$^-$ cells pulsed with the M$_{172}$ peptide. The lytic activity of responder cells was tested in a chromium-release assay either on RMAS-K^d or P1 AB$^-$ target cells that were pulsed or not with the peptide. Three mice out of ten displayed an anti-Mage-a2 CTL response (Table 1). After limiting dilution of lymphocytes stimulated in vitro for seven days, CTL clones were obtained from the three positive mice. They all lysed the cells pulsed with the peptide. A concentration of 100 pM was sufficient to produce 50% of the maximal specific lysis. However, these CTL clones were unable to lyse cells expressing Mage-a2 (data not shown).

To obtain CTL that recognized cells expressing the Mage-a2 sequence, the lymphocytes were stimulated and tested on P1.HTR.B7 cells, hereafter called P1.Mage-a2$^+$, which had been transfected with a truncated *Mage-a2* gene, and as a result produced a truncated protein (aa 1–200). Initial attempts to obtain P1.HTR transfectants expressing the entire *Mage* sequence failed, presumably because high expression of the *Mage* genes in selected cells stopped the growth of these cells, as we have observed with several *MAGE* genes. We therefore transfected cells with a construct where the sequence coding for the last 100 amino acids had been deleted, in the expectation that the toxicity would disappear. This produced transfectants with an adequate level of Mage-a2 expression. P1.HTR.B7 cells transfected with the selective pHMR plasmid alone, hereafter called P1.Mage-a2$^-$, were used as a negative control. Again, three mice out of ten injected showed a specific response (Table 2). The CTL populations derived from the responder animals lysed not only P1 AB$^-$ cells pulsed with the peptide but also P1.Mage-a2$^+$ transfected cells.

Mice immunized with Adeno.Mage-a2 (1–200)

To confirm the immunogenicity of the M$_{172}$ peptide and to increase the number of responding animals, we decided to turn to the adenovirus system. Its efficacy had been demonstrated in immunization experiments against antigen P815AB of mastocytoma P815 [50]. The difficulty encountered with the transfection of the entire *Mage-a2* gene into P1.HTR led us to prepare a recombinant adenovirus encoding the first 200 amino acids of Mage-a2. The use of a truncated sequence was also chosen because the function of the Mage protein is unknown and its biological activity not elucidated. The construct with an incomplete sequence instead of the complete gene minimized risks resulting from its expression in cells infected with the recombinant adenovirus. This Adeno.Mage-a2 (1–200) included the sequence encoding the M$_{172}$ peptide.

DBA/2 mice were injected once intradermally into the ears with 10^9 pfu Adeno.Mage-a2 (1–200) or with an adenovirus producing β-galactosidase as a negative

Fig. 1 Sequence of the *Mage-a2* open reading frame and of the corresponding protein. The antigenic Mage-a2$_{172-180}$/K^d peptide is shown in a *box*

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ATG GCT GAC TCC CAT AAC ACC CAA TAC TGC AGC CTC CAA GAG AGT GCT CAG GCC CAA CAG     60
 M   A   D   S   H   N   T   Q   Y   C   S   L   Q   E   S   A   Q   A   Q   Q      20

GAA TTA GAC AAT GAC CAG GAG ACC ATG GAG ACA TCA GAG GAG GAG GAA GAT ACC ACC ACC    120
 E   L   D   N   D   Q   E   T   M   E   T   S   E   E   E   E   D   T   T   T      40

TCA AAT AAA GTG TAT GGC AGT GGA ATA CCA AGT CCT CCC CAG AGT CCT CAG AGA GCC TAC    180
 S   N   K   V   Y   G   S   G   I   P   S   P   P   Q   S   P   Q   R   A   Y      60

TCT CCC TGT GTG GCA CTG GCC TCC ATC CCT GAT AGC CCA TCT GAG GAA GCT TCC ATC AAA    240
 S   P   C   V   A   L   A   S   I   P   D   S   P   S   E   E   A   S   I   K      80

GGA TCA GGG GGC CTG GAA GAC CCA CTT TAT TTG TTG CAC AAT GCA CAG AAC ACA AAG GTG    300
 G   S   G   G   L   E   D   P   L   Y   L   L   H   N   A   Q   N   T   K   V     100

TAT GAC TTG GTG GAC TTT CTG GTT TTA AAC TAT CAA ATG AAG GCA TTC ACT ACC AAA GCA    360
 Y   D   L   V   D   F   L   V   L   N   Y   Q   M   K   A   F   T   T   K   A     120

GAA ATG TTG GAA AGT ATT GGT AGA GAG TAT GAG GAG TAC TAC CCT CTG ATC TTT AGT GAG    420
 E   M   L   E   S   I   G   R   E   Y   E   E   Y   Y   P   L   I   F   S   E     140

GCC TCT GAG TGC TTG AAG ATG GTC TTT GGC CTT GAC ATG GTA GAA GTG GAC CCC TCT GTC    480
 A   S   E   C   L   K   M   V   F   G   L   D   M   V   E   V   D   P   S   V     160

CAC TCC TAT ATC CTT GTC ACT GCC CTG GGG ATC ACC TAT GAT GGC ATG ATG ACT GAT GTC    540
 H   S   Y   I   L   V   T   A   L   G   I   T   Y   D   G   M   M   T   D   V     180

CTG GGT ATG CCC AAG ACA GGT ATC CTC ATA GTC GTA CTG AGT GTC ATT TTC ATG AAG GGA    600
 L   G   M   P   K   T   G   I   L   I   A   V   L   S   V   I   F   M   K   G     200

AAC TAT GTC AGT GAG GAG ATT ATC TGG GAA ATG GTG AAT AAC ATA GGA TTG TGT GGT GGG    660
 N   Y   V   S   E   E   I   I   W   E   M   V   N   N   I   G   L   C   G   G     220

AGG GAT CCT TAC ATA CAT AAA GAC CCC AGG AAG CTC ATC TCT GAG GAG TTT GTG CAG GAA    720
 R   D   P   Y   I   H   K   D   P   R   K   L   I   S   E   E   F   V   Q   E     240

GGG TGC CTG AAA TAC AGG CAG GTG CCC AAT AGT GAT CCT CCT AGC TAT GGG TTC CTG TGG    780
 G   C   L   K   Y   R   Q   V   P   N   S   D   P   P   S   Y   G   F   L   W     260

GGC CCA AGG GCT TTT GCA GAA ACC AGC AAA ATG AAA GTC TTA CAG TTT TTT GCC AGC ATT    840
 G   P   R   A   F   A   E   T   S   K   M   K   V   L   Q   F   F   A   S   I     280

AAT AAG ACT CAT CCC AGA GCC TAC CCT GAA AAG TAT GCA GAG GCT TTG CAA GAT GAG ATA    900
 N   K   T   H   P   R   A   Y   P   E   K   Y   A   E   A   L   Q   D   E   I     300

GAC AGG ACC AAG GCC TGG ATC TTG AAC AGA TGC TCC AAC TCC TCT GAC CTG CTC ACA TTC    960
 D   R   T   K   A   W   I   L   N   R   C   S   N   S   S   D   L   L   T   F     320

TAG                                                                                963
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The box in the sequence encloses: `ACC TAT GAT GGC ATG ATG ACT GAT GTC` / `T Y D G M M T D V`

control. Fourteen days later, their splenocytes were recovered and stimulated with irradiated syngeneic splenocytes pulsed with the M$_{172}$ peptide or with irradiated P1.Mage-a2$^+$ transfected cells. After 7 days of stimulation with peptide pulsed cells, the lymphocytes from six out of ten mice injected with Adeno.Mage-a2 (1–200) showed lytic activity against the cells pulsed with peptide (Table 3). Lymphocytes of one mouse also lysed P1.Mage-a2$^+$ transfected cells. When stimulated with the P1.HTR.B7 cells transfected with the truncated *Mage-a2* gene, lymphocytes from nine out of ten mice lysed, with even higher efficacy, M$_{172}$ pulsed cells and four out of ten also recognized P1.Mage-a2$^+$ transfected cells (Table 3, Fig. 3A). No lytic activity was observed in mice injected with Adeno.βgal.

Some CTL clones were isolated from three mice whose splenocytes were stimulated for 7 days with irradiated P1.Mage-a2$^+$ cells. Some CTL clones, such as CTL.ASm5.10 or CTL.ASm6.10 shown in Fig. 4, were directed against the M$_{172}$ peptide as expected. Others, like CTL.ASm5.1 or CTL.ASm6.14, recognized specifi-

cally P1.Mage-a2$^+$ transfectants but not cells pulsed with the peptide (Fig. 4; data not shown). Neither did they recognize the K^d transfected B78H1 cells (see below, and data not shown). Transient cotransfections into human 293-EBNA cells of the truncated *Mage-a2* gene encoding the first 200 a.a. of the protein or various smaller fragments derived from it together with the *H*-2L^d gene, allowed us to identify a 129 bp fragment. This was able to stimulate several clones such as CTL.ASm.5.1, as measured by a TNF release assay. But so far, we have been unable to find a short peptide presented by L^d that was recognized by one of these CTL clones.

Mice immunized with Adeno.M$_{172}$

We wished to compare the efficacy of adeno.whole gene versus an adeno.minigene. We constructed an adenovirus containing a minigene producing the M$_{172}$ antigenic peptide, hereafter called Adeno.M$_{172}$.
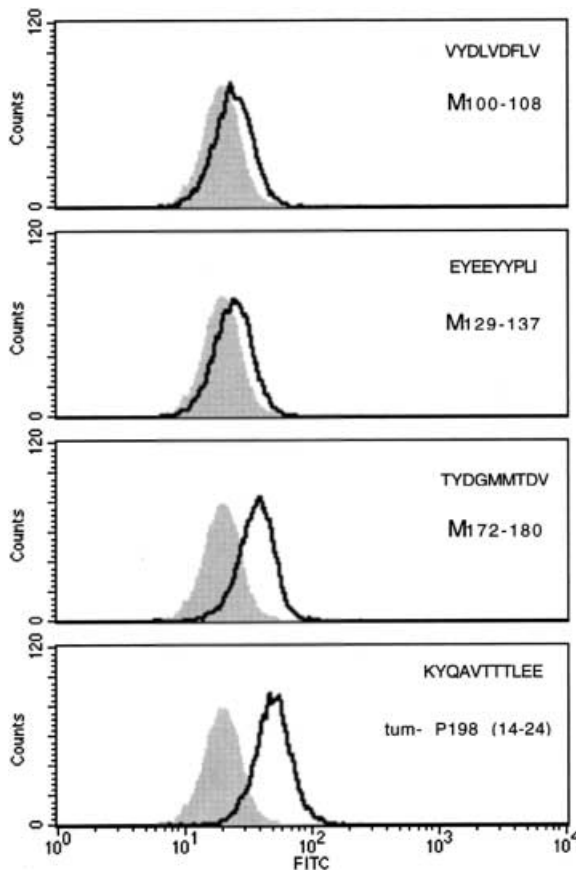
Fig. 2 Binding of three Mage-a2 peptides on K^d molecules present on RMA.S. The 3×10^5 RMA.S cells expressing low levels of class I H-2^b molecules and previously transfected with K^d were incubated with 50 μM peptide $M_{100–108}$, $M_{129–137}$ and $M_{172–180}$ in a medium containing 2% FCS. Control cells were incubated in medium alone. After 16 h, they were washed and incubated with 20 μl mAb SF1 1.1.1 (anti-K^d) at 4 °C for 45 min, then washed and incubated with 5 μl goat anti-mouse GAM-FITC. The shaded area represents control cells in the absence of peptide. Control peptide P198⁻.14–24 from mastocytoma P198 tum⁻ variant was used as a positive control since it is known to bind to K^d [35]

Some DBA/2 mice were injected once intradermally into the ears with 10^9 pfu Adeno.M_{172} or control Adeno.βgal. Fourteen days later, their splenocytes were stimulated in vitro with irradiated syngeneic spleen cells pulsed with the M_{172} peptide. After 7 days, their lytic activity was measured on P815 cells pulsed or not with the peptide and on P1.Mage-a2$^+$ and P1.Mage-a2$^-$ transfectants. Splenocytes from each of the ten mice injected with Adeno.M_{172} were able to lyse specifically target cells pulsed with the peptide (Table 3). Seven of them also recognized the P1.Mage-a2$^+$ transfected cells. The lytic activity of lymphocytes obtained from a few representative mice is illustrated in Fig. 3B.

After limiting dilution of the stimulated lymphocytes of mouse six (Fig. 3B), 11 CTL clones were obtained. They lysed specifically target cells pulsed with peptide M_{172} and transfected cells expressing the truncated *Mage-a2* gene. The lysis of one of them, CTL.AS.6.11, is shown in Fig. 5A.

In order to test whether these CTL clones recognized tumor cells expressing naturally *Mage-a2*, the *H*-2^b B78H1 cell line, which expresses *Mage-a2*, was cotransfected with pcDNAI-K^d and pSVtkneoβ plasmids. Geneticin transfectants were analyzed by FACS for the expression of K^d, using mAb SF1 1.1.1. A few clones expressing K^d were obtained. They were tested for recognition by CTL.AS.6.11. Because B78H1 is known to express low levels of class I molecules and is therefore a bad target for lysis, TNF assays were performed, after overnight incubation of the CTL with the target cells. The three B78H1 clones expressing K^d were recognized by the CTL (one representative clone is shown in Fig. 5B). Thus the CTL present in mice immunized with Adeno.M_{172} were able to recognize tumor cells that expressed the *Mage-a2* gene. With Adeno.M_{172}, we observed that the route of immunization was important, with a clear advantage for intradermal injection into the ears versus the intraperitoneal route (Fig. 6).

Table 1 CTL response of mice immunized with Mage-a2/K^d peptide and adjuvant[a] (numbers are % of specific lysis at the plateau of activity)

Mouse	Stimulation	Target cells[b]			
		RMAS-K^d M_{172} pulsed	RMAS-K^d not pulsed	P1 AB$^-$ M_{172} pulsed	P1 AB$^-$ not pulsed
Mouse 6	P1 AB$^-$	14	9	12	14
	P1 AB$^-$ M_{172} pulsed	58	11	64	8
Mouse 7	P1 AB$^-$	18	12	16	11
	P1 AB$^-$ M_{172} pulsed	47	3	52	11
Mouse 10	P1 AB$^-$	14	5	9	11
	P1 AB$^-$ M_{172} pulsed	25	10	21	16

[a] 10 DBA/2 mice were injected into each footpad with 25 μg of purified Mage-a2/Kd peptide (M_{172}) mixed with adjuvant SBAS-1c (1:1), four times at 14-day intervals. Two weeks after the last injection, the splenocytes were collected. Immune spleen cells were stimulated with irradiated P1. AB$^-$ cells pulsed or not with the peptide. After 7 days, they were tested in a 4 h ^{51}Cr assay on target cells pulsed or not with the peptide

[b] To avoid non-specific lysis, P1.AB$^-$ cold cells were added to target cells at 50–70:1

Table 2 CTL response of mice immunized with Mage-a2/K^d peptide and adjuvant[a] (numbers are % of specific lysis at the plateau of activity)

Mouse	Stimulation	Target cells[b]			
		P1.Mage-a2$^+$	P1.Mage-a2$^-$	P1 AB$^-$ M$_{172}$ pulsed	P1 AB$^-$ not pulsed
Mouse 4	P1 AB$^-$	4	4	12	19
	P1.Mage-a2$^+$	63	19	80	8
Mouse 6	P1 AB$^-$	7	14	8	7
	P1.Mage-a2$^+$	60	1	77	3
Mouse 9	P1 AB$^-$	7	11	9	2
	P1.Mage-a2$^+$	29	22	46	12

[a] 10 DBA/2 mice were injected into each footpad with 25 µg of purified Mage-a2/Kd peptide (M$_{172}$) mixed with adjuvant SBAS-1c (1:1), four times at 14-day intervals. Two weeks after the last injection, the splenocytes were collected. Immune spleen cells were stimulated with irradiated P1. AB$^-$ cells or with irradiated P1.HTR.B7 cells transfected with the truncated *Mage-a2 (1–200)* gene. After 7 days, they were also tested on P1.Mage-a2$^+$ and P1.Mage-a2$^-$ transfected cells
[b] To avoid non-specific lysis, P1.AB$^-$ cold cells were added to target cells at 50–70:1

Table 3 CTL response induced by various immunogens (*A* P1.ABCD$^-$ cells pulsed with 10 µM M172 peptide for 2 h, *B* P1.HTR.B7 cells transfected with a truncated *Mage-a2* gene)

Immunization with	Splenocytes of mice stimulated with	Lysis (no. responding mice/total) on		
		Transfectant Mage-a2$^+$	Cells pulsed with peptide M172–180	Non-specific or no lysis
Peptide + adjuvant	A	0/10	6/20	14/20
	B	2/10	3/10	7/10
Adeno.Mage-a2 (1–200)	A	1/10	6/10	4/10
	B	4/10	9/10	1/10
Adeno.M172	A	7/10	10/10	0/10

DBA/2 injected once with :

A Adeno.Mage-a.2 (1-200)

Mouse 2   Mouse 3   Mouse 6

B Adeno.M$_{172}$

Mouse 1   Mouse 3   Mouse 6

% Cr-51 specific release — Ratio

◆ P815 + M$_{172}$ ▲ P1.Mage-a2$^+$ ● P1.Mage-a2$^-$ ■ P815 no peptide
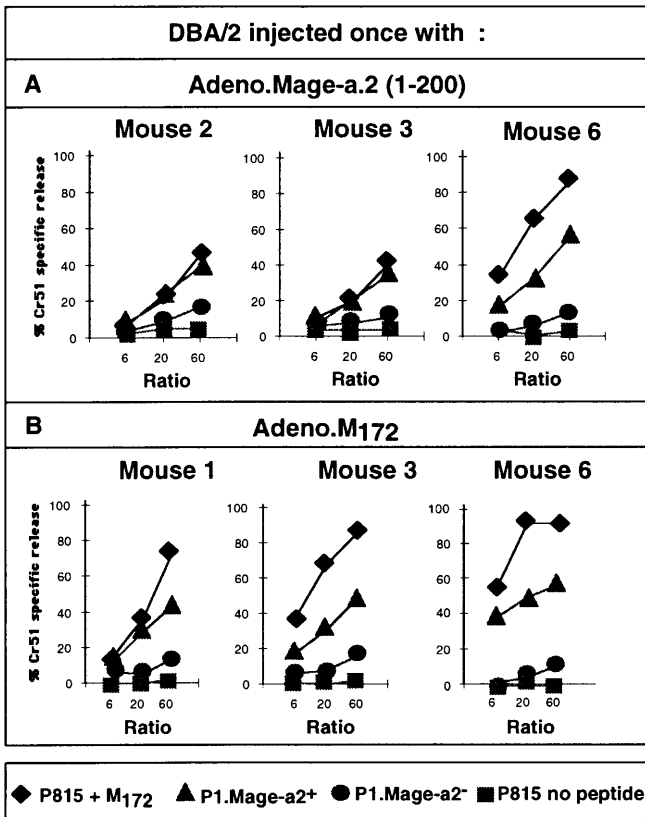
◄
Fig. 3 Response of mice injected once with Adeno.Mage-a2 (1–200) (**A**) or with Adeno.M$_{172}$ (**B**). DBA/2 mice were injected intradermally in the ears with 10^9 pfu. After 14 days, (**A**) their splenocytes were stimulated with irradiated P1.Mage-a2$^+$ cells or (**B**) irradiated syngeneic spleen cells, previously pulsed with 10 µM peptide M$_{172}$ for 2 h. Seven days later, they were incubated at various ratios with ^{51}Cr labeled P1 ABCD$^-$ cells pulsed with M$_{172}$ peptide (◆) or not (■), and with labeled P1.Mage-a2$^+$ (▲) and P1.Mage-a2$^-$ (●) transfected cells. The released radioactivity was measured after 4 h

Discussion

On the basis of the sequence of gene *Mage-a2*, a mouse homologue of the human *MAGE-A* genes, we have identified a peptide that binds to *H*-2K^d. Using this peptide to immunize mice, we were able to obtain CTL that lysed cells pulsed with the peptide. The antigenic peptide, Mage-a2$_{172–180}$, is clearly processed by tumor cells, as CTL that were isolated from animals immunized with a recombinant Mage-a2 adenovirus and restimulated in vitro with the peptide recognized not only peptide-pulsed cells but also tumor cells expressing naturally the Mage-a2 protein. In contrast, CTL clones obtained from mice immunized with peptide and adjuvant and similarly stimulated in vitro with cells pulsed with peptide recognized only cells pulsed with the peptide, probably because of their low avidity. This has also been observed for human antigenic peptides selected for their ability to bind to frequent class I molecules [19, 51] (P. van der Bruggen, personal communication).

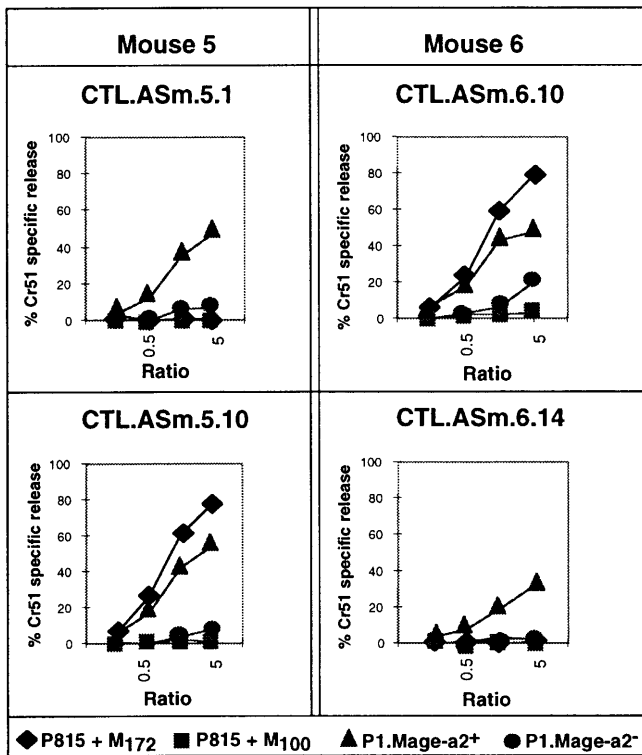## DBA/2 injected with Adeno.Mage-a.2 (1–200)

Fig. 4 Lysis by anti-Mage-a2 CTL clones. CTL clones were isolated by limiting dilution from DBA/2 mice injected with Adeno.Mage-a2 (1–200). They were incubated at various ratios with four labeled target cells: P1 ABCD$^-$ cells pulsed during the labeling with 10 μM M_{100} peptide (■) or M_{172} peptide (♦), transfected P1.Mage-a2$^+$ cells (▲) and P1.Mage-a2$^-$ cells (●). After 4 h, the supernatant was collected and released radioactivity counted

Fig. 5 A Lysis by CTL clone CTL.AS. 6.11 directed against M_{172} peptide presented by K^d. This clone was isolated by limiting dilution, from a mouse immunized with Adeno.M_{172}. For the ^{51}Cr release assay, CTL were incubated at various ratios with the four labeled target cells mentioned in the legend of Fig. 3. After 4 h, the supernatant was collected and released radioactivity was counted. **B** Recognition by CTL.AS.6.11 of B78H1 melanoma cells transfected with K^d. CTL.AS.6.11 cells (2×10^3) were incubated with B78H1 cells (2×10^4) preincubated for 24 h in 25% of secondary MLC supernatant. Clone-17 expressed the K^d molecule, clone-23 did not. P1.HTR.B7 transfectants that expressed or not the truncated Mage-a2 were used as controls. After 24 h, the TNF contained in the supernatant was measured by incubation with 2×10^4 WEHI-164.cl13 cells. Colorimetric MTT assay was performed after 24 h [40]

Mice immunized with a recombinant adenovirus coding for the first 200 amino acids of the *Mage-a2* protein mounted a strong CTL response: some of the CTL were directed against the Mage-a2$_{172-180}$ antigenic peptide, while others were directed against another epitope presented by L^d. We have been unable to define the latter epitope, even though we have restricted the encoding sequence to amino acid 147–189 of Mage-a2, by transfection experiments. None of the overlapping peptides covering this sequence were able to render P815 cells sensitive to lysis. Examples of "abnormal" antigens that could not be predicted from the sequence of the main protein product of known genes have been described. Alternative open reading frame (ORF) were found to produce new epitopes like TRP-1 [49], LAGE-1 and NY-ESO-1 [1, 48], M-CSF (M. Probst-Kepper and B. Van den Eynde, in preparation), or mouse LP-BM5 retrovirus [27]. Anti-sense transcription was also shown to produce an antigenic epitope [45]. However, this does not seem to be applicable to our case as peptides encoded by alternative ORF were not recognized. But other sources of unexpected epitopes exist such as a post-translational modification, as observed for the
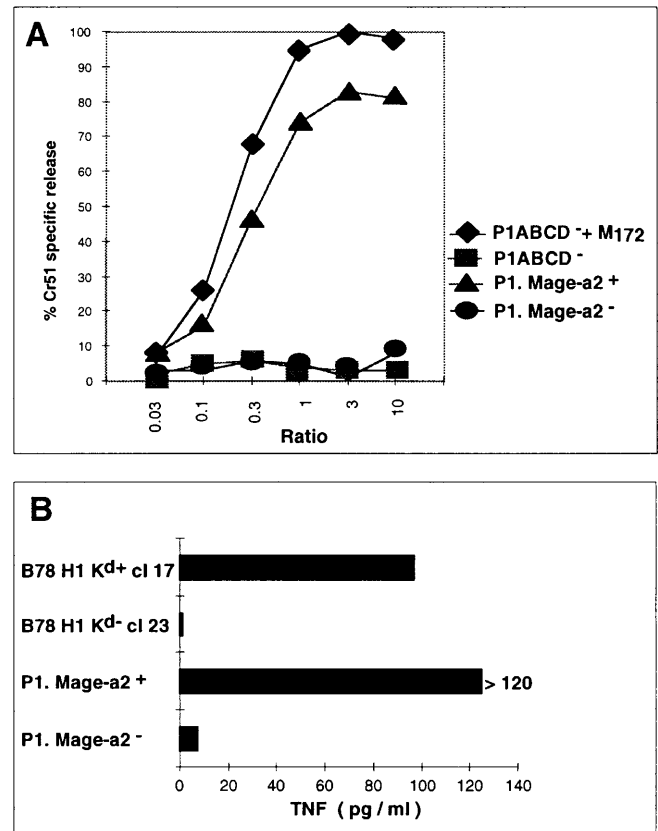
tyrosinase peptide restricted by HLA-A2 [37], where an asparagine was modified to an aspartic acid.

Various modalities of immunization have been tested previously for mouse antigen P815AB, an important tumor-specific antigen of mastocytoma P815. These modalities were compared for their effectiveness in inducing CTL responses. P1 A antigenic peptide was tested with various adjuvants. No CTL response was obtained by combining the P1A peptide with complete or incomplete Freund adjuvant or Alum (C. Uyttenhove, personal communication). P1A peptide preceded by a sequence targeting the peptide to the endoplasmic reticulum [29] was also ineffective. To induce T helper cells, P1A peptide was injected in adjuvant together with two CD4$^+$ epitopes recognized by *H-2*^{d} mice, the *Plasmodium berghei* circumsporozoite peptide Pb20–39
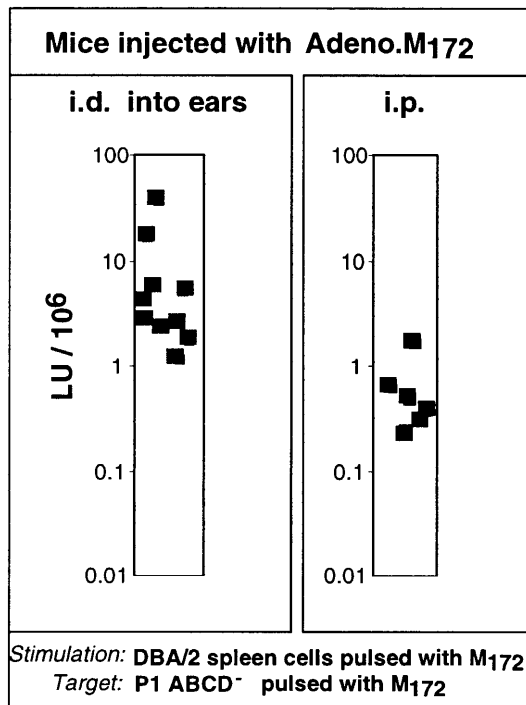
Fig. 6 Response of DBA/2 mice injected with Adeno.M_{172} using various routes of immunization. Recombinant adenoviruses (10^9 pfu) were injected intradermally (*i.d.*) into the ears or intraperitoneally (*i.p.*). Fourteen days later, their splenocytes were stimulated for 7 days with irradiated syngeneic spleen cells pulsed with 10 µM M_{172} peptide. The ^{51}Cr release assay was performed on P1 ABCD$^-$ cells pulsed with the peptide. Response is given in LU/10^6 cells. Each point represents the lytic activity of one individual mouse

[28] and the tetanus toxin peptide tt 947–967 [12]. No enhancing effect on CTL responses was observed. However, the adjuvant combination of QS21 and MPL (SBAS-1c) proved to be effective. Upon four injections of peptide P1A mixed with this adjuvant, CTL responses were observed in 30–60% mice [36]. It was shown that the injection of IL-12 around the time of peptide-adjuvant injection significantly improved the CTL response [36]. But the best modality of immunization, observed so far, involves recombinant viruses. After a single injection of a recombinant adenovirus coding for the P1A peptide, a specific CTL response was observed in nearly all the mice [50], probably because the recombinant adenoviruses constitute an efficient system for the introduction of foreign genes into cells of the infected host.

In agreement with the results observed with P1A, when encoded into a recombinant adenovirus, the Mage-a2 epitope induced a strong CTL response. The route of immunization appears to be important for the induction of a response, intradermal injection into the ears being superior to intraperitoneal injection. This was also shown for the immunization of Balb/c mice with tumor cell extracts containing gp96 [6] or subcutaneous versus intravenous injection of dendritic cells

pulsed with the TRP2 peptide [13]. The adenovirus carrying the minigene coding for the epitope was superior to the virus containing a large part of the *Mage-a2* gene. This may be due to the fact that processing by the proteasome is not required for the nonapeptide encoded by the minigene whereas it is required for the 200 amino acids protein [30].

Our results demonstrate that mice can mount a strong immune response against Mage antigens. This system will be used to explore new modalities of vaccination that can be applied to human therapeutic vaccination of patients with Mage-related antigens.

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