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Synthetic Peptides Derived from the Melanocyte-stimulating Hormone Receptor MC1R Can Stimulate HLA-A2-restricted Cytotoxic T Lymphocytes That Recognize Naturally Processed Peptides on Human Melanoma Cells[1]

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ABSTRACT

Human melanoma-specific HLA-A2 restricted CTLs have recently been shown to recognize antigens expressed by melanoma lines and normal melanocytes, including Melan-A/Mart-1, gp100, gp75, and tyrosinase. Herein, we define HLA-A2-restricted CTL epitopes from a recently cloned melanocortin 1 receptor (MC1R), which belongs to a new subfamily of the G-protein-coupled receptors expressed on melanomas and melanocytes. Thirty-one MC1R-derived peptides were selected on the basis of HLA-A2-specific motifs and tested for their HLA-A2 binding capacity. Of a group of 12 high or intermediate HLA-A2 binding peptides, three nonamers, MC1R244 (TILLGIFFL), MC1R283 (FLALIICNA), and MC1R291 (AIIDPLIYA), were found to induce peptide-specific CTLs from peripheral blood mononuclear cells of healthy HLA-A2+ donors after repeated *in vitro* stimulation with peptide-pulsed antigen-presenting cells. The CTLs raised against these three HLA-A2+-restricted peptides could recognize naturally processed peptides from HLA-A2+ melanomas and from Cos7 cells cotransfected with MC1R and HLA-A2. CTLs induced by the MC1R291 peptide (but not induced or induced only to a very low extent by the other two MCR1 peptide epitopes) showed cross-reactions with two other members of the melanocortin receptor family, which are more broadly expressed on other tissues. Taken together, our findings have implications in relation both to autoimmunity and immunotherapy of malignant melanomas.

INTRODUCTION

The cloning of genes encoding human melanoma-associated antigens and the definition of CTL peptide epitopes derived from them has stirred considerable interest among immunologists and clinicians. One category of tumor-specific molecules, exemplified by the *MAGE* gene family of antigens (1, 2), is derived from genes transcriptionally activated in several types of tumors but not normally expressed in adult tissues. Another category of tumor-specific melanoma antigens, of which fewer examples exist, are those derived from mutated genes, such as one derived from mutated cyclin-dependent kinase 4 (3). The third and major category of melanoma antigens, against which T-cell responses in melanoma patients have been demonstrated most frequently, is differentiation antigens. These include Melan-A/Mart-1, gp100, gp75, and tyrosinase (4–7), which encode peptide CTL epitopes presented by HLA-A2 molecules (8, 9). These antigens are also expressed on normal melanocytes in the skin and on melanocyte-like cells in the retina, and therefore, they should be regarded as true "self" antigens. CTLs reacting with these antigens can often be demonstrated in patients with advanced melanomas. These results have been interpreted as evidence for the breaking of immunological tolerance to normal cellular proteins, perhaps due to overexpression of these molecules on tumor cells.

MC1R[3] is expressed on the cell membrane of cells of melanocytic origin, including melanoma cells (10–18). This receptor belongs to a new subfamily among the G-protein-coupled receptors, which are ligands for the MCs α-MSH and adenocorticotropin, which are peptide hormones. α-MSH and adenocorticotropin have established roles in pigmentation and adrenal function, respectively (19–26), and also have been shown to exert other biological effects (19). More specifically, molecular cloning has identified five structurally different human MC receptors (MC1R, 2R, 3R, 4R, and 5R; Refs. 19, 22, and 25–26). These MC receptors are involved in the regulation of cell proliferation and differentiation, pigmentation, and metastatic potential of melanomas (12–15). Stimulation of MC receptors results in increased levels of cytosolic calcium, accumulation of cAMP, and activation of protein kinase C (16, 17).

Herein, we have identified peptide epitopes from MC1R that can bind to HLA-A2, and we have demonstrated that three nonamer peptides binding with high or intermediate affinity can reproducibly generate peptide-specific CTLs from PBMCs of healthy HLA-A2+ donors. These peptide-specific CTLs could also recognize HLA-A2+ melanoma cells expressing MC1R or Cos7 cells transfected with MC1R and HLA-A2, demonstrating that the MC1R-derived peptides are naturally processed and presented by MHC class I on the surface of melanoma cells.

MATERIALS AND METHODS

Peptide Synthesis and HLA-A2.1 Binding Assay. MC1R peptides were identified on the basis of the HLA-A2.1 binding motif using a computer algorithm (27). Nonamer peptides were synthesized by a solid phase method by using a multiple peptide synthesized and purified by high-performance liquid chromatography as described previously (5, 6). Peptide binding to HLA-A2 was measured as described (28). Briefly, various doses of the test peptides (ranging from 100 μM to 1 nM) were coincubated together with the 5 nM radiolabeled HBVc18–27 (FLPSDYFPSV) peptide and HLA-A2.1 heavy chain and β_2-microglobulin for 2 days at room temperature in the presence of a mixture of protease inhibitors. The percentage of MHC-bound radioactivity was determined by gel filtration, and the ID_{50} was calculated for each peptide.

Cell Lines. BEmel; BLmel; DLmel; FMSmel; AKmel; the DF-derived sublines DFBmel, DFWmel (HLA-A2+), and H1264mel; ACBmel; and BM-Wmel (A2−) are melanoma cell lines established at the Microbiology and Tumor Biology Center of the Karolinska Institute. 397mel (A2−) was kindly provided by Dr Y. Kawakami (National Cancer Institute, Bethesda, MD).

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[3] The abbreviations used are: MC1R, melanocortin 1 receptor; MC, melanocortin; MSH, melanocyte-stimulating hormone; APC, antigen-presenting cell; PBMC, peripheral blood mononuclear cell; PBL, peripheral blood lymphocyte; NDP-MSH, NIe^4DPhe^7-α-MSH; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; TNF, tumor necrosis factor; mAb, monoclonal antibody; TIL, tumor infiltrating lymphocyte; LCL, lymphoblastoid cell line.

FM3Dmel and FM55.M1mel (A2+) were kindly provided by Dr. J. Zeuthen (Cancer Society, Copenhagen, Denmark). The T2 line is a TAP-defect cell line derived from the human T cell leukemia/B cell LCL hybrid 174 (29). C1R-A2 is a class I-defective cell line transfected with HLA-A2 (30).

Transfection of COS-7 Cells and Functional Expression of MC1R. COS-7 cells were cultured in RPMI 10% FCS in a 6-well plate. Seventy % confluent cultures were transfected with 2 μg of DNA and 25 μl of Lipofectin (Costar Corp. and Life Technologies, Inc.) in 1 ml serum-free medium Opti-MEM (Costar Corp. and Life Technologies, Inc.). After overnight incubation, the serum-free medium was removed and RPMI 1640 with 10% FCS was added to the plates, which were cultured for an additional 48 h. Transfected cells or melanoma cells were washed with 0.3 ml of binding buffer (MEM with Earle's salts, 25 nM HEPES, pH 7.0, 0.2% BSA, 1 mM 1,10-phenanthroline, 0.5 mg/liter leupeptin, and 200 mg/liter bacitracin) and then incubated at 37°C for 2 h with 0.3 ml binding buffer containing 24,000 cpm of [^{125}I]NDP-MSH. NDP-MSH was labeled with ^{125}I and purified as described (19) to a specific activity of 8.6×10^4 Ci/mol. Nonspecific binding was determined in the presence of 1 μM unlabeled NDP-MSH. The radioactive binding medium was removed, and cells were washed with 0.3 ml of cold binding buffer. Cells were then scraped off in 0.1 M NaOH, and the bound radioactivity was counted on a γ counter.

Primary CTL Induction Using Synthetic Peptide. PBMCs from HLA-A2+ healthy donors were separated from PBLs by centrifugation on Ficoll-Hypaque (Pharmacia UpJohn, Uppsala, Sweden) gradients. Peptide-specific CTL lines were generated as follows: 8×10^6 PBMCs plus 2×10^6 peptide-pulsed autologous PBLs (fresh APCs) were plated at day 0 in 75 cm^3 bottles (Costar Corp. and Life Technologies, Inc.) in 5 ml of AIM-V medium (Life Technologies, Paisley, United Kingdom) containing 5 IU/ml IL-2 (kindly supplied by Dr. P. Simon, DuPont Merck Pharmaceutical Co.). Fresh APCs were obtained by incubating PBMCs with 5 μg/ml peptide at 26°C for 2 h and then removing free peptides. At the same time, 3×10^6 PBMCs were incubated for 3 h at 37°C in 75-cm^3 flasks. Nonadherent cells were removed, and the adherent cells were cultured in 3 ml of AIM-V medium containing 100 ng/ml GM-CSF (specific activity, 1.11×10^7 colony-forming units/mg protein; Molgramostim, Leukomax). The APCs were fed twice a week with medium containing GM-CSF and maintained for 4 weeks. The CTLs were re-stimulated weekly with peptide-loaded GM-CSF cultured monocytes and maintained in medium containing 5 IU/ml IL-2. After four stimulations, the CTLs were tested for specificity using cytotoxic assay or TNF release assay.

Cytotoxic Assay and TNF Release Assay. To analyze peptide recognition, T2 cells, autologous LCL cells, or C1R-A2 cells were incubated for 2 h at 26°C together with 2 μg/ml peptide, washed, and used as target cells in ^{51}Cr release assays or as stimulator cells in TNF release assays. Cytotoxic tests were performed by incubating ^{51}Cr-labeled target cells (1×10^6) with effector cells at various E:T ratios at 37°C for 4 h. Supernatants were harvested, and radioactivity was determined using a gamma counter. The percentage of ^{51}Cr release was calculated according to the following formula:

$$\% \text{ lysis} = 100 \times \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}}$$

To induce TNF production, 5×10^4 CTLs from cell lines or 1×10^4 CTLs from clones were incubated together with 5×10^4 stimulator cells, and the supernatants were collected after 24 h. TNF content was determined by testing the cytotoxic effects of the supernatants on WEHI 164 clone 13 cells in a 3-(4,5-dimethyllythiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay (31).

Statistical Analysis. Differences in TNF induction between triplicates were compared using Student's *t* test and were considered statistically significant when *P*s were <0.05.

RESULTS

Identification of Peptides from MC1R That Bind to HLA-A2. The amino acid sequence of MC1R was screened for the most probable HLA-A2 nonapeptide epitopes using a computer program that takes into account the presence of main A2-specific anchor residues, as well as specific secondary anchor residues (27). The 31 peptides that obtained the highest scores were synthesized and tested for binding to purified HLA-A2 molecules (Table 1). The results from the binding assay showed that 3 of the tested peptides showed a high binding affinity for HLA-A2 (<50 nM required to achieve 50% inhibition), 7 were intermediate HLA-A2 binders (50–500 nM to achieve 50% inhibition), and the remaining 21 bound HLA-A2 only weakly (500 nM to 50 μM to achieve 50% inhibition).

Induction of Primary CTL Responses to MC1R-derived Peptides. The 12 highest MHC binding peptides, which included all high and intermediate binders and two of the low binding peptides, were tested for their capacity to elicit HLA-A2-restricted CTLs. PBMCs from at least four different healthy HLA-A2+ donors were stimulated with the synthetic peptides and autologous APCs (GM-CSF cultured PBMC-derived monocytes) for at least four rounds of stimulation over a period of 4–6 weeks. Of the three MHC high binders, one (MC1R283, FLALIICNA) was able to induce specific CTLs against peptide-pulsed, HLA-A2-transfected C1R or T2 mutant cell lines in PBMCs from three of five healthy donors (Fig. 1*A*). Two of the seven intermediate A2 binders, MC1R244 (TILLGIFFL; Fig. 1*B*) and MC1R291 (AIIDPLIYA; Fig. 1*C*), were also able to elicit peptide-specific CTLs from PBMCs of the majority (four of five for MC1R291 and four of six for MC1R244) of the normal donors tested. For all three peptides, these CTLs were specific because they did not kill the corresponding nonpulsed target cell or target cells pulsed with irrelevant HLA-A2 binding peptides or the NK target K562 (Fig. 1). The peptide specificity of these CTLs was observed even in the presence of a 20-fold excess of K562 cells. None of the other nine MC1R-derived peptides tested were able to generate CTLs from PBMCs of at least three different healthy HLA-A2+ donors (data not shown).

Table 1 *Relative binding affinity of MC1R-derived peptides to HLA-A2.1*

Peptide name	Sequence	A*0201 (nM)[a]
MC1R99	LLLEAGALV	24
MC1R251	FLCWGPFFL	37
MC1R283	FLALIICNA	39
MC1R9	RLLGSLNST	167
MC1R45	FLSLGLVSL	206
MC1R92	VLETAVGLL	238
MC1R291	AIIDPLIYA	286
MC1R79	CLALSDLLV	357
MC1R52	SLVENALVV	429
MC1R244	TILLGIFFL	455
MC1R49	GLVSLVENA	532
MC1R43	GLFLSLGLV	535
MC1R13	SLNSTPTAI	633
MC1R189	LLCLVVFFL	676
MC1R285	ALIICNAII	893
MC1R200	LVLMAVLYV	1210
MC1R85	LLVSGSNVL	1408
MC1R210	MLARACQHA	1639
MC1R196	FLAMLVLMA	2083
MC1R25	GLAANQTGA	2381
MC1R223	RLHKRQRPV	2941
MC1R98	ILLLEAGAL	4762
MC1R258	FLHLTLIVL	5000
MC1R245	ILLGIFFLC	5556
MC1R131	SLCFLGAIA	10000
MC1R105	ALVARAAVL	11111
MC1R157	TLPRARRRV	25000
MC1R236	GLKGAVTLT	50000
MC1R304	ELRRTLKEV	>50000
MC1R84	DLLVSGSNV	>50000
MC1R23	QLGLAANQT	>50000

[a] Concentration of tested peptides required to inhibit 50% of the binding to HLA-A2.1 of 0.5 nM standard peptide (HBVc18–27; see "Materials and Methods"). According to affinity data, peptides are arbitrarily defined as highly (50% inhibition at <50 nM), intermediately (50% inhibition at 50–500 nM), and weakly (50% inhibition at >500 nM) binding peptides.

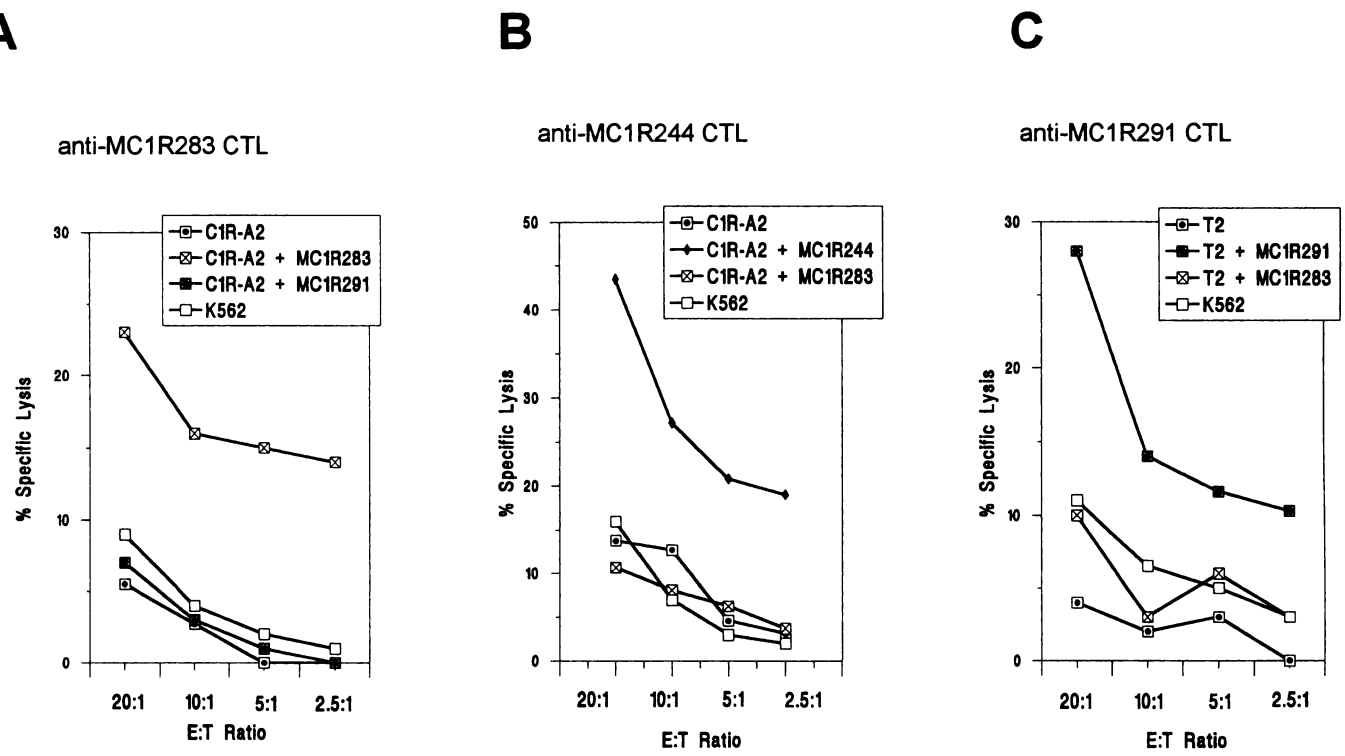

Fig. 1. Induction of a primary CTL response to MC1R-derived peptides after four weekly stimulations. CTLs were produced as described in "Materials and Methods" against MC1R283 peptide (FLALIICNA; *A*), MC1R244 peptide (TILLGIFFL; *B*), and MC1R291 peptide (AIIDPLIYA; *C*) and tested against peptide-pulsed targets in an standard ^{51}Cr release assay as described. Similar results were obtained at least three times with independently produced lines for each peptide.

MHC Class I Binding and CTL Cross-Reactivity of Nonapeptides Derived from the Peptide Sequence Homologues to MC1R244, MC1R283, and MC1R291 of MC2R, MC3R, MC4R, and MC5R. Five different members of the MC receptor family have been cloned, each of which has distinct tissue localization (19, 22, 24–26). For each of the three MC1R CTL epitopes identified, peptide homologues corresponding to the homologous sequence of the MC2R, 3R, 4R, and 5R family members were synthesized and tested for HLA-A2.1 binding affinity. The MC1R244 homologues derived from MC2R, MC3R, and MC4R bound with very low affinity to purified HLA-A2. In contrast, the MC5R analogue actually bound with a 30-fold increased affinity relative to the parent MC1R244 peptide (Table 2). All MCR283 homologue peptides bound only very weakly to HLA-A2. Finally, the MC2R, MC3R, and MC4R homologues of the MC1R291 epitope all showed a reduction from 80% to 50% of binding relative to the parentally derived peptide, whereas the MC5R homologue of MC1R291 demonstrated a 3-fold increase in its HLA-A2 binding capacity (Table 2).

Table 2 *Relative binding affinity of MCR peptides to HLA-A2.1*

Peptide	Sequence	A*0201 nM[a]	Ratio to MC1R[b]
MC1R244	TILLGIFFL	96	1.00
MC2R244	TILLGVFIF	4545	0.02
MC3R244	TILLGVFIF	4545	0.02
MC4R244	TILIGVFVV	4545	0.02
MC5R244	TMLLGVFTV	3	30.80
MC1R283	FLALIICNA	98	1.0
MC2R283	NGMLIMCNA	16667	0.006
MC3R283	YLVLINCNS	2174	0.045
MC4R283	YLILIMCNS	6250	0.016
MC5R283	YLILIMCNS	6250	0.016
MC1R291	AIIDPLIYA	238	1.0
MC2R291	AVMDPFIYA	758	0.31
MC3R291	SVIDPLIYA	1190	0.20
MC4R291	SIIDPLIYA	455	0.52
MC5R291	SVMDPLIYA	83	2.90

[a] Concentration of tested peptides required to inhibit 50% of the binding to HLA-A2.1 of 0.5 nM standard peptide (HBVc18–27; see "Materials and Methods").
[b] Ratio to the binding activity of the MC1R peptide.

We next analyzed whether the CTLs elicited against the MC1R peptides 244 and 283 could recognize C1R-A2 targets pulsed with the analogous peptides derived from the other MCR family members. An anti-MC1R244 CTL line showed cytotoxic activity against C1R-A2 pulsed with MC1R244 peptides, but only a very low lytic activity against MC2/3R peptide and did not recognize cells pulsed with the others members of the MCR family, including the high binding MC5R244 peptide (Fig. 2*A*; Table 2). Similarly, C1R-A2 loaded with peptides derived from the MC2R, MC3R, MC4R, and MC5R sequences homologous to MC1R283 all failed to be recognized by MC1R283-specific CTLs (Fig. 2*B*). The results are consistent with the poor binding capacity of the MC2R283, MC3R283, MC4R283, and MC5R283 peptides (Table 2).

MC1R-specific CTLs Can Specifically Recognize Cos7 Cells Cotransfected with MC1R and HLA-A2. To analyze whether the MC1R peptide-specific CTLs could recognize naturally processed MC1R-derived peptides, Cos7 cells were cotransfected with plasmids containing the MC1R, MC3R, MC4R, or MC5R genes together with a HLA-A2 containing vector. The double transfectants transiently expressed both proteins, as measured by binding of radiolabeled NDP-MSH peptide to MC1R (Fig. 3) and by flow cytometric analysis of HLA-A2 expression (data not shown). The MC2R plasmid was not used because the cloned cDNA is very poorly expressed.[4]

A CTL line (CTL12) specific for the MC1R244 peptide and a CD8+ clone derived from it (C3) could recognize Cos7 cells cotransfected with MC1R and HLA-A2 but not untransfected Cos7 or Cos7 transfected only with HLA-A2 (Fig. 4*A*). There was only a low level of specific TNF release when the same CTLs were stimulated with

[4] V. Chhajlani, unpublished results.

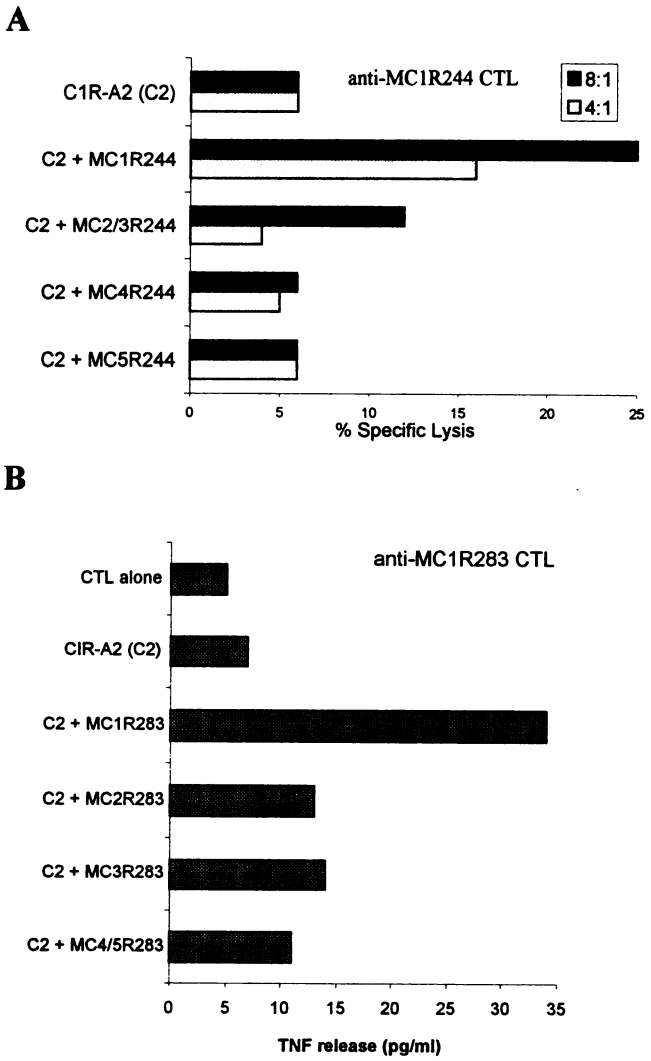

Fig. 2. Specific recognition of CTLs against MC1R and other MCR subtype-derived peptides. CTLs elicited against MC1R244 peptide (TILLGIFFL; *A*) or MC1R283 peptide (FLALIICNA; *B*) were tested by standard ^{51}Cr release assay or TNF release assay against synthetic peptides derived from the homologous sequences of the different MCR subtypes.

A2-transfected Cos7 cotransfected with MC3R, MC4R, or MC5R, which differ significantly (P = 0.006, 0.019, and 0.008, respectively, for the AM line, and P = 0.0015, 0.007, and 0.008, respectively, for the C3 clone) from the TNF release observed with the MC1R transfectant. This is in line with the poor capacity of MC1R244-specific CTLs to recognize peptide homologues from the same receptors (see Fig. 2*A*). Furthermore, a CD8+ T cell clone (AM) isolated from the CTLs raised against MC1R291 efficiently recognized the Cos7 transfectants coexpressing the MC1R and HLA-A2 but did not recognize the Cos7 cells transfected with MC1R or HLA-A2 alone (Fig. 4*B*). The same MC1R291-specific T cell clone also cross-reacted with the Cos7 cells coexpressing MC3R and MC4R with HLA-A2 but not the ones expressing MC5R (Fig. 4*B*).

MC1R-specific CTLs Can Specifically Recognize HLA-A2+ Melanoma Cells Expressing MC1R. Thirteen melanoma lines, along with 2 colon carcinomas, 1 ovarian carcinoma, and 2 EBV-transformed B cell lines, were screened for the expression of MC1R (Table 3). Eight of the 13 melanoma lines, and none of the other lines, expressed MC1R.

All of the tested melanomas that expressed MC1R and that were HLA-A2+ (n = 6) were recognized by MC1R-specific CTLs, whereas none of the three MC1R− HLA-A2+ lines and neither of the two MC1R-expressing HLA-A2− lines were recognized (Table 3). For example, MC1R244 peptide-specific CTLs recognized the MC1R-expressing HLA-A2+ melanoma lines BLmel and BEmel, and the tumor-induced TNF production could be blocked by mAb HB54, which recognizes HLA-A2 (Fig. 5*A*). Neither an A2− melanoma cell line [MC1R− (397mel)], an A2+ colon cancer cell line (SW480) negative for MC1R, nor the NK-sensitive cell line K562 was recognized by the CTLs (Fig. 5*A*). Similarly, the 0351CTL line elicited by MC1R283 peptide-specific recognized T2 cells pulsed with MC1R283 peptide and FM55mel cells expressing both HLA-A2 and MC1R but not the A2+, MC1R− FM3Dmel, T2 alone or loaded with influenza peptide, the colon cancer line SW480, or the K562 line (Fig. 5*B*). This cytotoxic activity was HLA-A2 restricted because it could be blocked by mAb HB54, which recognizes HLA-A2 (Fig. 5*B*).

CTLs elicited by the MC1R291 peptide showed cytotoxic activity against both BEmel and BLmel cell lines but did not recognize autologous EBV-transformed B cell lines (BE LCL and BL LCL) or K562 (Fig. 5*C*). A CD8+ clone (C8) derived from this line also recognized BEmel expressing MC1R but not the MC1R−, HLA-A2+ DLmel or the MC1R+, HLA-A2− H1264 or ACBmel (Fig. 5*D*). The capacity to recognize the BEmel was blocked by anti-HLA-A2 mAb (Fig. 5*D*).

We therefore conclude that all three MC1R-derived peptides characterized here are expressed and recognized on HLA-A2+ melanomas.

DISCUSSION

In the present report, we demonstrate that MC1R encodes epitopes that are recognized by human CTLs and that are presented as naturally processed peptides on human melanomas. Several MC1R-derived HLA-A2 motif-containing peptides have been characterized for their MHC binding affinity and immunogenicity in primary *in vitro* CTL cultures using PBMCs from normal donors. As a result, three epitopes have been defined, and the corresponding CTLs have been characterized in terms of their capacity to recognize various melanoma tumor lines and MC1R transfectant cell lines and in terms of their capacity to recognize peptides from other highly homologous members of the

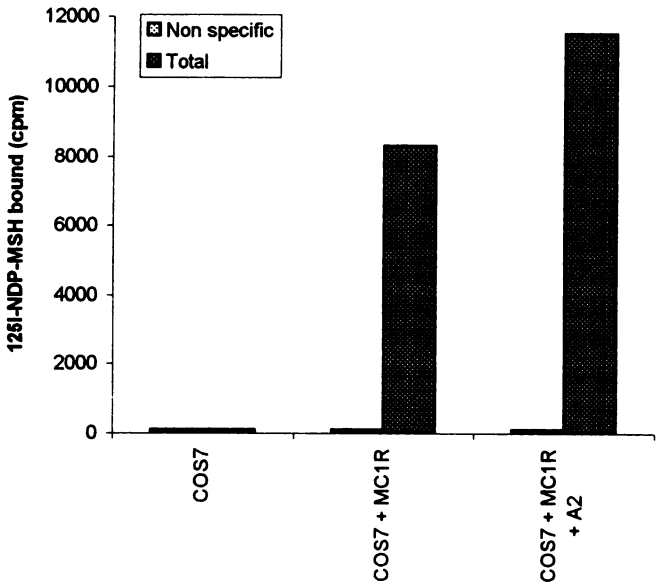

Fig. 3. Expression of MC1R on COS-7 cells after transfection with the MC1R gene. Cells were incubated with [^{125}I]NDP-MSH analogue and washed, the radioactivity was counted, and data were analyzed as described in "Materials and Methods."

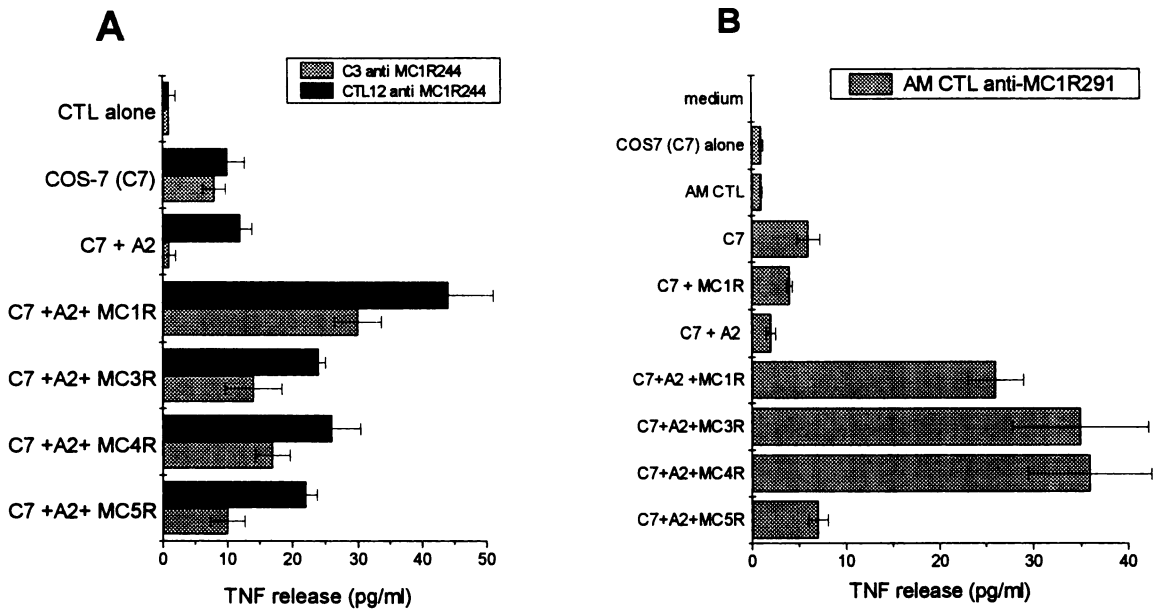

Fig. 4. Specific recognition of CTLs against Cos-7 cells cotransfected with the HLA-A2 gene and MC1R gene or the other MCR-subtype genes. A CTL12 line elicited against MC1R244 peptide (TILLGIFFL) and a CTL clone (C3) derived from this line (*A*) and a CD8+ T cell clone (AM) derived from a CTL elicited against MC1R291 peptide (FLALIICNA; *B*) were tested by TNF release assay against COS-7 cells cotransfected with HLA-A2 and the genes from the homologous sequences of the different MCR subtypes as described.

MC receptor family. Our results suggest that MC1R is a true tumor-associated antigen of potential relevance and utility for the study and immunotherapy of human malignant melanoma.

Recent evidence supports the existence of several new HLA-A2.1- and HLA-A3-restricted melanoma epitopes unrelated to known antigens within the melanoma-specific T-cell repertoire (32, 33). These new antigens were detected by CTLs isolated from lymph nodes of melanoma patients, and their expression was detected on neoplastic but not on cultured normal cells of melanocytic lineage. These results suggest that several tumor antigens may still await identification and characterization. In the present study, we decided to investigate whether MC1R contains class I-restricted CTL epitopes, which are potentially useful in the immunotherapy of malignant melanomas.

We selected the MC1R protein for the present study because early reports using binding of radiolabeled peptides (10, 20) demonstrated that this receptor is present on the surface of melanoma cells, and RNA coding for MC1R could only be detected in melanoma cells (19). It is also interesting to note that the MC1R protein could not be detected in melanocytes from normal skin by immunohistochemistry, but it could easily be visualized on skin with melanoma tumor growth (18), indicating that melanoma cells have an increased expression of MC1R compared to normal melanocytes.

The binding assays performed herein confirm that MC1R is expressed on the majority (8 of 13) of melanomas, whereas it is not present in 2 colon carcinomas, 1 ovarian carcinoma, or 2 EBV-transformed B cell lines. Low levels of MC1R expression, however, could be detected in the testis and ovary by immunohistochemistry using MC1R-specific mAbs,[5] demonstrating that small amounts of the MC1R protein are synthesized by tissues other than those of the melanocytic lineage, a finding relevant to the possible use of MC1R-specific CTLs in immunotherapy for melanomas. The relative tissue-specific expression of the MC1R protein, therefore would place it in the same category as the tissue-specific Melan-A/Mart-1, gp100, gp75, and tyrosinase antigens.

Various methods have been used to define antigens, including genetic studies based on genomic or cDNA expression libraries (4–7) and biochemical studies based on high-performance liquid chromatography-derived peptide fractions (34). Tumor-specific CTLs, raised from patient TILs by culture in IL-2 (5, 6) or from patient PBMCs by repeated stimulation with the autologous tumor (4, 7), have been critical reagents for these studies. Herein, we demonstrate the usefulness of an alternative method, which is not constrained by the availability of such CTLs and thereby is not limited to the analysis of immunodominant epitopes recognized by CTLs from cancer patients during the course of their disease. According to this method, HLA-A2-binding peptides derived from the tumor-associated antigen of interest were identified, and their capacity to stimulate peptide- or melanoma-specific CTLs from PBMCs of healthy donors was tested. Although not used previously for the identification of new melanoma antigens, this approach was used for the identification of epitopes from tumor antigens, such as p53 and HER2/neu, expressed in other

Table 3 *Anti-MC1R specific CTLs only recognize MC1R-expressing HLA-A2+ melanomas*

Cell line	HLA haplotype	Cell line	Sensitivity to MC1R specific CTL[a]	Expression of MC1R[b]
BLmel	A2+	Melanoma	+	+
BEmel	A2+	Melanoma	+	+
DLmel	A2+	Melanoma	–	–
DFBmel	A2+	Melanoma	+	+
DFWmel	A2+	Melanoma	+	+
FM55.M1mel	A2+	Melanoma	+	+
FM3D	A2+	Melanoma	–	–
FMSmel	A2+	Melanoma	+	+
AKmel	A2+	Melanoma	–	–
397Mel	A2–	Melanoma	–	–
BMWmel	A2–	Melanoma	–	–
H1264Mel	A2–	Melanoma	–	+
ACBmel	A2–	Melanoma	–	+
SW480	A2+	Colon carcinoma	–	–
SW620	A2+	Colon carcinoma	–	–
OVA	A2+	Ovarian carcinoma	–	–
BE LCL	A2+	LCL	–	–
BL LCL	A2+	LCL	–	–

[a] Tested by ^{51}Cr release assay and/or TNF release assay as described in "Materials and Methods." All tumors were tested against at least two CTL lines specific for MC1R291, MC1R244, and MC1R283.

[b] The cell lines were tested twice for MCR expression by binding assay with radiolabeled NDP-MSH (see "Materials and Methods").

[5] Unpublished results.

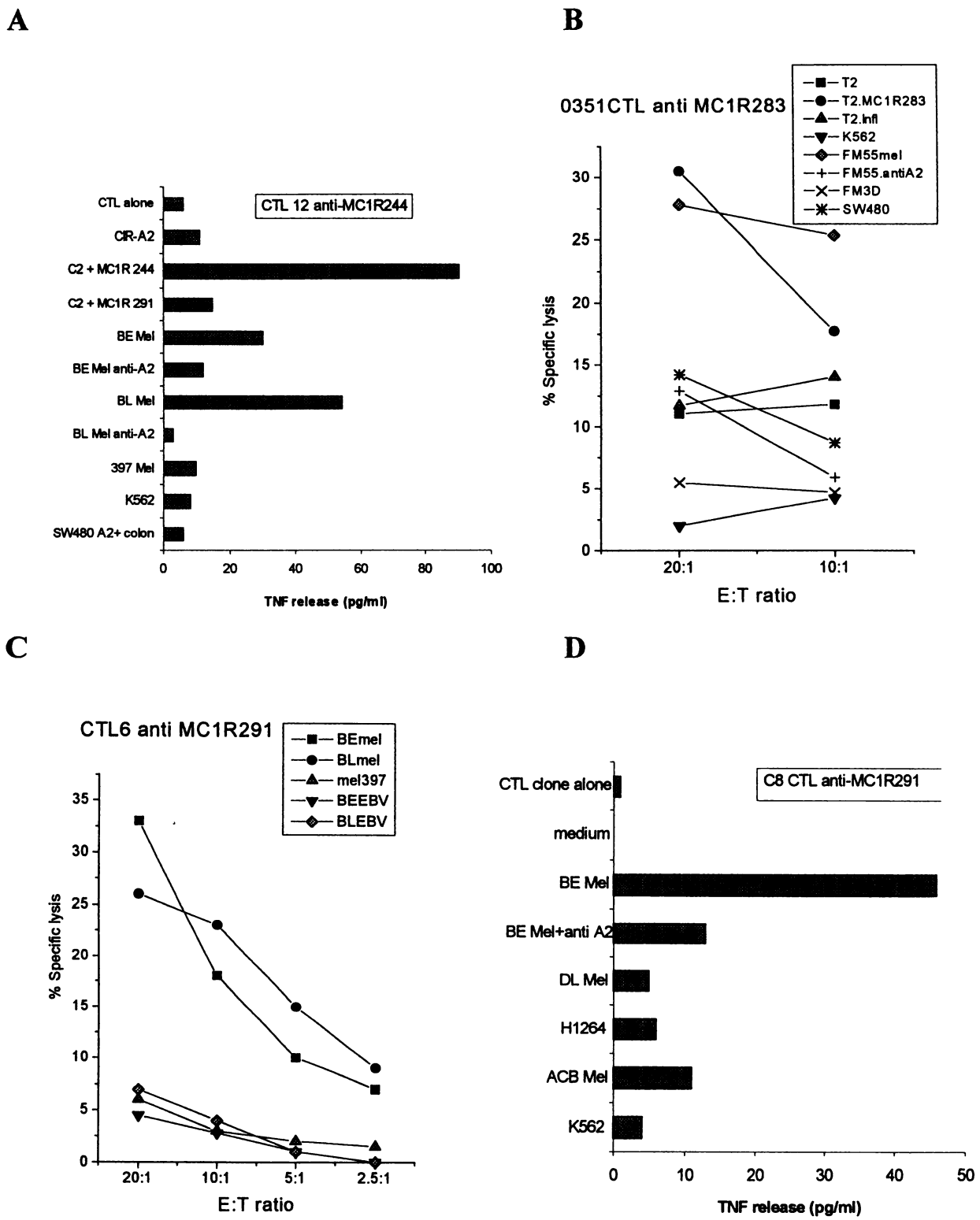

Fig. 5. CD8+ T cells elicited against MC1R synthetic peptides can recognize HLA-A2+ melanoma cell lines expressing the MC1R. An anti-MC1R244 CTL line (*A*), an anti-MC1R283 CTL (*B*), and anti-MC1R291 CTL line and clone C8 (*C* and *D*) were tested by TNF release assay (*A* and *D*) or a cytotoxicity assay (*B* and *C*) for specific activity against melanoma lines. Each CTL line was tested at least twice.

tumor types (35, 36). It has also been used for the definition of HLA-A2-restricted epitopes in melanoma antigens isolated by the genetic or biochemical approach (8, 9).

Sequencing pooled MHC class I-eluted peptides correlates the predominance of certain amino acids at defined positions in peptides with high MHC binding affinity (37, 38). For HLA-A2.1 the second position (P2) from the amino terminus most frequently contains leucine or methionine, and a valine is predominant at P9 (or P10 in 10mers). Additional studies using purified HLA molecules and quantitative binding assays revealed several other residue types that were not immediately obvious from the analysis of pooled naturally processed peptides (39). This led to the definition of extended HLA

motifs, which include not only the canonical anchors revealed by pooled sequencing but often related acceptable or "tolerated" residues as well. Of the three MC1R-derived HLA-A2-associated peptide epitopes characterized here, only one (MC1R283) contains a canonical residue at P2, whereas the remaining two contain a tolerated residue (isoleucine) at this position. Furthermore, none of the three MC1R epitopes contain a canonical residue at P9. Thus, these results demonstrate the usefulness of extended motifs to map actual CTL epitopes from tumor-associated antigens.

It has previously been demonstrated that >90% of peptides recognized by CTLs specific for viral Ags were high or intermediate-affinity HLA binders, whereas only few cases of low or negative binding peptides have been identified (40). Of the MC1R-derived peptides tested that could induce specific CTLs, one (MC1R283) belonged to the group of highly binding peptides, whereas the other two (MC1R291 and MC1R244) were intermediate binders. Because we have not tested more than two of the low affinity binders, we cannot exclude the possibility that some of these may also be able to induce CTLs with our sensitization protocol. The MC1R283 peptide was able to induce specific CTLs from PBLs obtained from three of five healthy donors, demonstrating that T cells are not tolerant against this high-affinity binding self-epitope. The highest HLA-A2 binding peptides from gp100 and Melan-A/Mart-1 have also been shown to be CTL epitopes (9), so for at least these melanoma-associated self antigens there appears to be no correlation between high MHC binding and induction of tolerance.

The ability of healthy donors to mount a CTL response against MC1R suggests that TCRs able to recognize these self proteins are present in the normal T-cell repertoire. This response may represent an example of an *in vitro* induction of a primary response or, alternatively, a weak secondary response following sensitization *in vivo* by peptides expressed on melanocytes or other MC1R-expressing cells.

The three MC1R-derived peptide epitopes that we have characterized in this report appear to be highly immunogenic in the majority of healthy donors. This is in contrast to HLA-A1-restricted MAGE-1-specific CTL lines, which could be generated in only one of five melanoma patients and only by *in vitro* stimulation of TILs, not of PBMCs with peptide-pulsed autologous EBV-transformed B cells (41). In this regard, the MC1R-derived peptide epitopes are comparable to Melan-A/Mart-1 epitope (28–35), which can generate CTLs from PBMCs of a large proportion of healthy donors (42). It remains to be seen to what extent we will be able to generate MC1R-specific CTLs from HLA-A2+ melanoma patients PBMCs or TILs, and if so, to what extent it may require a lower number of Ag re-stimulations as compared to using PBMCs from healthy donors, as has been shown to be the case for Melan-A/Mart-1-derived CTL responses (42).

T cells induced *in vitro* with APCs pulsed with peptides identified by their ability to bind to MHC class I alleles have frequently been shown to recognize peptide-pulsed target cells but not target cells presenting naturally processed epitopes (43, 44). Presumably, this is due to the low levels of epitope presentation by the latter. It is of particular interest, therefore, that CTL lines and clones generated against the three MC1R-derived epitopes characterized here were able to recognize MC1R+ HLA-A2+ melanomas as well as Cos7 cells transiently transfected with MC1R and HLA-A2 genes. One possibility is that the melanomas and the transfected Cos7 cells tested may express high levels of processed antigen. Alternatively, our method for CTL generation, which involves the use of autologous PBMCs grown in serum-free medium with GM-CSF as APCs for peptide pulsing, may favor the outgrowth of high-affinity CTL precursors bearing the capacity to recognize cell surface epitopes expressed at low density.

The MC family of receptors consists of five closely related members. Of the CTL lines raised against the three MC1R-derived CTL epitopes described here, none cross-reacted against the MC5R receptor, which is expressed on a broad variety of human tissues (23). The CTL line elicited against the MC1R244 peptide showed only a very low degree of cross-reactivity against the other MCR family members, at the border of being significant, whereas the MC1R291-reactive CTL line also recognized the MC3R and MC4R homologous peptides. The MC3R molecule is expressed in human brain and heart (23, 24), whereas MC4R is predominantly expressed in brain and is also weakly expressed in the pituitary gland (23, 25). Although we failed to see cytotoxic activity of the MC1R-specific CTL lines against autologous EBV-transformed lymphoid lines and against HLA-A2+ colorectal and ovarian carcinomas, additional experiments screening for the ability of the MC1R-specific CTL lines to recognize various human tissues would be necessary before considering any therapeutical trial on melanoma patients.

In conclusion, the results presented herein demonstrate that the MC1R antigen encodes for HLA-class I-restricted CTL epitopes and that these epitopes are presented as naturally processed antigenic peptides on human melanomas. These results underline the potential usefulness of MC1R-derived epitopes in the characterization of naturally occurring antimelanoma CTL responses, as well as in the development of epitope-based immunotherapies.

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