Published Online: 1 March, 1999 | Supp Info: http://doi.org/10.1084/jem.189.5.871
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Brief Definitive Report

Melanoma Cells Present a MAGE-3 Epitope to CD4+ Cytotoxic T Cells in Association with Histocompatibility Leukocyte Antigen DR11

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Summary

In this study we used TEPITOPE, a new epitope prediction software, to identify sequence segments on the MAGE-3 protein with promiscuous binding to histocompatibility leukocyte antigen (HLA)-DR molecules. Synthetic peptides corresponding to the identified sequences were synthesized and used to propagate $CD4^+$ T cells from the blood of a healthy donor. $CD4^+$ T cells strongly recognized MAGE-$3_{281–295}$ and, to a lesser extent, MAGE-$3_{141–155}$ and MAGE-$3_{146–160}$. Moreover, $CD4^+$ T cells proliferated in the presence of recombinant MAGE-3 after processing and presentation by autologous antigen presenting cells, demonstrating that the MAGE-3 epitopes recognized are naturally processed. $CD4^+$ T cells, mostly of the T helper 1 type, showed specific lytic activity against HLA-DR11/MAGE-3–positive melanoma cells. Cold target inhibition experiments demonstrated indeed that the $CD4^+$ T cells recognized MAGE-$3_{281–295}$ in association with HLA-DR11 on melanoma cells. This is the first evidence that a tumor-specific shared antigen forms $CD4^+$ T cell epitopes. Furthermore, we validated the use of algorithms for the prediction of promiscuous $CD4^+$ T cell epitopes, thus opening the possibility of wide application to other tumor-associated antigens. These results have direct implications for cancer immunotherapy in the design of peptide-based vaccines with tumor-specific $CD4^+$ T cell epitopes.

Key words: MAGE-3 • $CD4^+$ epitopes • melanoma • tumor vaccines • adoptive immunotherapy

The importance of $CD4^+$ T lymphocytes in antitumor immunity has been clearly demonstrated in animal models. $CD4^+$ T cells exert helper activity for the induction and maintenance of antitumor $CD8^+$ T cells (1–7), but they may also have an effector function either by indirect mechanism against MHC class II–negative tumors, via macrophages activation (for a review, see reference 1), or by direct mechanism against MHC class II–positive tumors (6, 7).

Recently, the requirement of cognate $CD4^+$ T cell help for optimal induction of antitumor $CD8^+$ CTLs was demonstrated (8). Vaccination with a specific viral T helper epitope, but not with an unrelated T helper epitope, resulted in protective immunity against MHC class II–negative, virus-induced tumor cells. Moreover, simultaneous vaccination with the tumor-specific T helper and CTL epitopes resulted in strong synergistic protection.

In humans, evidence for a role of $CD4^+$ T cells in antitumor immunity comes from the study of tumor-infiltrating lymphocytes, which revealed the presence of both $CD8^+$ and $CD4^+$ T cells at the tumor site (9, 10), and from detection in the sera of neoplastic patients of antibodies directed against tumor antigens (for a review, see reference 11). However, in recent years research on T cell immunity against human tumors has focused mainly on identification of $CD8^+$ HLA class I–restricted CTL responses. To date tyrosinase, a tissue-specific antigen expressed in normal and neoplastic cells of melanocytic lineage, is the only melanoma-associated antigen demonstrated as a specific target

J. Exp. Med. © The Rockefeller University Press • 0022-1007/99/03/871/06 $2.00
Volume 189, Number 5, March 1, 1999 871–876
http://www.jem.org

for $CD4^+$ melanoma-reactive T cells (12, 13) and for which $CD4^+$ T cell epitopes have been identified (14).

Characterization of the $CD4^+$ T cell epitope repertoire on other tumor-associated antigens, especially those that are tumor-specific and shared among tumors of several histotypes (for a review, see reference 15), would contribute decisively to improve the efficacy of peptide-based immunization protocols in neoplastic patients.

MAGE-3 is a tumor-specific antigen encoded by a gene expressed in a high proportion of melanomas and in several other tumor histotypes (head and neck squamous cell carcinomas, bladder carcinomas, lung carcinomas and sarcomas) and not in normal tissues, with the exception of testis and placenta (for a review, see reference 15). $CD8^+$ CTLs from melanoma patients recognize HLA class I–restricted MAGE-3 epitopes (15), and clinical trials with synthetic peptides corresponding to HLA-A1 and/or -A2 MAGE-3 binding sequences are ongoing in patients affected by melanoma and other neoplastic diseases (15). Therefore, MAGE-3 is an excellent candidate protein to study the antitumor $CD4^+$ T cell response. This protein has an intracytoplasmic localization (16), making its presentation on MHC class II molecules unlikely or difficult. However, it has been clearly shown that the MHC class II pathway can present endogenous cellular peptides (17–19), and peptides eluted from purified HLA-DR molecules of the melanoma cell line FM3 contained peptides derived from processing of cytoplasmic proteins (20).

In this study, we used a new T cell epitope prediction software (TEPITOPE; reference 21, and our manuscript in preparation) to identify MAGE-3 sequences with promiscuous HLA-DR binding characteristics. Synthetic peptides corresponding to five identified sequences were used to propagate $CD4^+$ T cells from the blood of a healthy donor. We show that $CD4^+$ T cells are MAGE-3 specific and recognize naturally processed sequence segment(s). Moreover, $CD4^+$ T cells are cytolytic and recognize MAGE-$3_{281-295}$ in association with HLA-DR11 on melanoma cells.

Materials and Methods

T Cell Epitope Prediction. TEPITOPE, a new T cell epitope prediction software, is a Windows™ application that enables the identification of (a) class II ligands binding in a promiscuous or allele-specific mode, and (b) the effects of polymorphic residues on class II ligand specificity (21, and our manuscript in preparation). 25 quantitative matrix-based HLA-DR motifs, covering the majority of class II ligand specificity, are incorporated in TEPITOPE (22, and our manuscript in preparation) and provide the basis for various algorithms included in the software package. Starting from any protein sequence, the algorithm permits the prediction and parallel display of ligands for each of the 25 HLA-DR alleles. To predict MAGE-3 $CD4^+$ T cell epitopes, we loaded the protein sequence into the software looking for promiscuous peptide regions. We set the TEPITOPE prediction threshold at 5% (21) and picked peptide sequences predicted to bind at least 50% of the HLA-DR molecules incorporated in the software.

DR–Peptide Binding Assay. Peptide interactions with detergent-solubilized DR molecules were measured using an ELISA-based high-flux competition assay (23). HLA-DR molecules were isolated from the following human lymphoblastoid cell lines (LCL): DR1 (DRB1*0101) from HOM-2, DR3 (DRB1*0301) from WT49, DR4 (DRB1*0401) from PREISS, DR5 (DRB1*1101) from SWEIG, DR7 (DRB1*0701) from EKR, and DR8 (DRB1*0801) from BM9. DR2 (DRB1*1501) was isolated from the L cell transfectant L466.1. The molecules were affinity purified using the mAb 1-1C4 (24) as described (25). Peptide competition assays were conducted to measure the ability of unlabeled peptides to compete with a biotinylated indicator peptide for binding to purified DR molecules. The following biotinylated indicator peptides were used: $GFKA_7$ for DR1 and DR7; $GIRA_2YA_4$ for DR2; $LAYDA_5$ for DR3; UD4 for DR4 (26); TT 830–843 for DR5; and $GYRA_6L$ for DR8. The biotinylated indicator peptide and HLA-DR molecules were incubated with 10-fold dilutions (0.001–100 mM) of the unlabeled competitor peptides (peptides corresponding to the MAGE-3 predicted sequences). To determine relative peptide binding affinity, the promiscuous $HA_{307-319}$ peptide from influenza hemagglutinin (27) was included in each competition assay. The relative binding data of the unlabeled competitor peptides were expressed as inhibitory concentration (IC_{50}), i.e., the concentration of competitor peptide required to inhibit 50% of binding of the biotinylated indicator peptide.

Peptide Synthesis. Synthetic peptides corresponding to MAGE-$3_{141-155}$, MAGE-$3_{146-160}$, MAGE-$3_{156-170}$, MAGE-$3_{171-185}$, and MAGE-$3_{281-295}$ sequences were manufactured on a 9050 Millipore synthesizer. The purity of the peptides was evaluated by reverse-phase HPLC and electron spray mass spectrometry. Synthetic peptides were lyophilized and then reconstituted in DMSO at 2 mg/ml concentration and diluted in PBS as needed.

Cloning and Expression of rMAGE-3. Full-length MAGE-3 coding sequences were inserted into expression vector pET16b (Novagen), allowing the production of the NH_2 terminus 10-histidine tail as described (16). Production and purification of the recombinant fusion protein on nickel column were monitored by SDS-PAGE and Coomassie blue staining.

Propagation of $CD4^+$ T Cells. The five synthetic peptides corresponding to the MAGE-3 sequences most promiscuous for HLA-DR binding (see Table I) were pooled (hereafter MAGE-3 pool) and used to stimulate the PBMCs of a healthy donor whose HLA type, identified by standard serologic typing, is A1, A2/B41, B52/DR11, as described (28). In brief, 20×10^6 PBMCs were cultivated for 7 d in RPMI 1640 (GIBCO BRL) supplemented with 10% heat-inactivated human serum (Technogenetics), 2 mM l-glutamine, 100 U/ml penicillin, 50 μg/ml streptomycin (Biowhittaker) (TCM) containing the MAGE-3 pool (1 μg/ml of each peptide). The reactive lymphoblasts were isolated on a Percoll gradient (28), further expanded in T cell growth factor (Lymphocult; Biotest Diagnostic Inc.), and restimulated at weekly intervals with the same amount of antigen plus irradiated (4,000 rad) autologous PBMCs as APCs.

Flow Cytometry. Cytofluorimetric analyses were performed on a FACStarPlus® (Becton Dickinson). The following mAbs were used: anti-CD4–PE and anti-CD8–FITC (Becton Dickinson), D1.12 (purified from an anti-MHC class II hybridoma supernatant), and 57B (described in reference 16). FITC-rabbit anti–mouse Ig antibody (DAKO) was used as second-step reagent in indirect immunofluorescence stainings. Staining for intracytoplasmic MAGE-3 expression was performed as described (29). Intracytoplasmic staining for cytokine expression was performed

using the anti–INF-γ and anti–IL-4 mAbs, following the manufacturer's instructions (Sigma).

Proliferation Assay. $CD4^+$ T cells and autologous irradiated PBMCs were diluted in TCM to 2×10^5/ml and 2×10^6/ml, respectively, and plated in triplicate in 96 round-bottomed well plates (100 μl of $CD4^+$ T cells and 100 μl of APCs). The cells were stimulated with different concentrations of MAGE-3 pool (0.05, 0.1, 0.5, 1, and 5 μg/ml), each peptide (10 μg/ml), and different concentrations of rMAGE-3 protein (5, 10, and 20 μg/ml). Triplicate wells with $CD4^+$ T cells alone and APCs alone were used as controls. Three wells with $CD4^+$ T cells plus APCs did not receive any stimulus in order to determine the basal growth rate (the blank). In inhibition experiments, different concentrations of mAb L243 or an isotype-matched irrelevant mAb (0.25 and 0.5 mg/ml) were added in triplicate wells of $CD4^+$ cells plus APCs stimulated with MAGE-3 pool (5 μg/ml) or MAGE-$3_{281-295}$ (10 μg/ml). After 3 d, the cultures were pulsed for 16 h with $[^3H]$TdR (1 mCi/well, 6.7 Ci/mol; Amersham Pharmacia Biotech). The cells were collected with a Titertek multiple harvester (Skatron, Inc.), and the thymidine incorporated was measured in a liquid scintillation counter. The percentage of inhibition was calculated as follows: [(cpm without mAb − cpm with mAb)/(cpm without mAb)] × 100.

Cytotoxicity Assay. $CD4^+$ T cells were tested for specific lytic activity in a standard 4-h ^{51}Cr-release assay as described (30). The following targets were used: melanoma cells (SK-Mel 28, HT144, OI TC described in reference 29, and MD TC established in our laboratory from a cutaneous metastasis), and LCL. The HLA-DR type of target cells, identified by molecular or serologic typing, was SK-Mel 28 (DR*04*13), HT144 (DR*04*07), OI TC (DR*01*11), MD TC (DR*04*11), LCL (DR11). In cold target competition assays, unlabeled target cells (cold targets) were seeded in plates at serial ratios of hot-to-cold target cells. Effector $CD4^+$ T cells and ^{51}Cr-labeled target cells (hot targets) were then added, and cytotoxicity was assessed as described above. Percentage inhibition was calculated as follows: [(% specific lysis without cold target − % specific lysis with cold target)/(% specific lysis without cold target)] × 100.

Results and Discussion

10 synthetic peptides corresponding to sequence segments predicted by TEPITOPE to form promiscuous MAGE-3 $CD4^+$ T cell epitopes were synthesized, and their binding to purified molecules of 7 widely diffuse HLA-DR alleles was verified. Based on the results of the competition binding assays, 5 (i.e., the sequences with the greatest degree of promiscuity) of the 10 predicted sequences were chosen for further experiments (Table I). The five synthetic peptides were pooled (MAGE-3 pool) and used to stimulate the PBMCs of a healthy donor. T cells were 94% $CD4^+$ after 1 wk of culture (not shown), and could be propagated in long-term culture by weekly restimulation with the MAGE-3 pool in the presence of autologous irradiated PBMCs. Reactivity of $CD4^+$ T cells was tested in microproliferation assays (Fig. 1): the cells responded vigorously to the MAGE-3 pool (Fig. 1 A), even at low concentrations (100–500 ng/ml). Reactivity to the individual peptides forming the pool was also periodically investigated (Fig. 1 C): the $CD4^+$ T cells recognized predominantly the peptide corresponding to MAGE-$3_{281-295}$

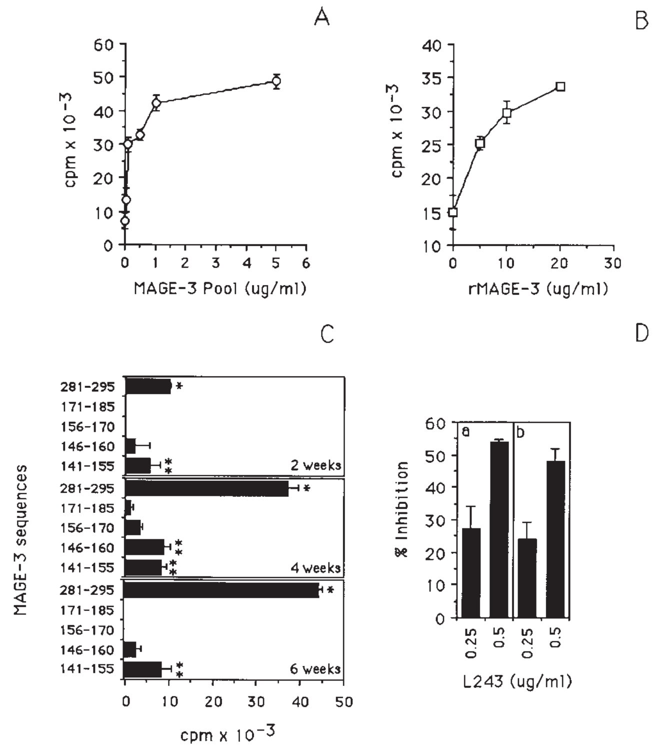

Figure 1. Proliferative activity of $CD4^+$ T cells stimulated with MAGE-3 pool measured in 2-d microproliferation assays. The data are representative of $n = x$ experiments, and are means of triplicate determinations ± SD. (A) Responses to MAGE-3 pool (0.01, 0.5, 0.1, 0.5, 1, and 5 μg/ml; $n = 6$). (B) Responses to rMAGE-3 protein (5, 10, and 20 μg/ml; $n = 3$). (C) Responses to the individual synthetic peptides forming the MAGE-3 pool (10 μg/ml; $n = 7$) at different weeks of propagation. The blank (i.e., the basal level of proliferation of $CD4^+$ T cells in the presence of APCs only) was subtracted and was as follows: 2 wk, 30,866 ± 1,115; 4 wk, 7,106 ± 2,201; and 6 wk, 21,838 ± 2,767. Responses significantly higher than the blanks are indicated as $^*P < 0.001$ and $^{**}P < 0.025$ (determined by unpaired, one-tailed Student's t test). (D) Response to MAGE-3 pool (5 μg/ml; $n = 5$) (a) and to peptide corresponding to sequence 281–295 (b), in the presence of different doses of L243 mAb (0.25 and 0.5 μg/ml). The blank was 1,251 ± 444; the proliferation of $CD4^+$ T cells in the presence of MAGE-3 pool was 28,191 ± 373; and the proliferation in the presence of sequence 281–295 was 22,504 ± 141.

and, although to a much lower but significant extent, the peptides corresponding to the overlapping sequences MAGE-$3_{141-155}$ and MAGE-$3_{146-160}$. All three sequences recognized by the $CD4^+$ T cells showed a high binding affinity to purified DR11 molecules (see Table I). Reactivity to MAGE-$3_{281-295}$ increased during the propagation of the line (Fig. 1 C). The proliferative activity of $CD4^+$ T cells in the presence of MAGE-3 pool (Fig. 1 D, a) or MAGE-$3_{281-295}$ (Fig. 1 D, b) was inhibited by addition in culture of different concentrations of L243 mAb (Fig. 1 D), demonstrating that the recognition of MAGE-3 sequences was HLA-DR restricted. We next tested the $CD4^+$ T cells for cross-reactivity with the native protein (Fig. 1 B). $CD4^+$ T cells strongly recognized the rMAGE-3 protein after processing and presentation by autologous APCs, demonstrating that the synthetic sequences recognized by the $CD4^+$ T cells indeed formed naturally processed epitopes.

Table I. *Determination of HLA-DR Binding of MAGE-3 Synthetic Peptides Corresponding to Sequences Predicted to Form Promiscuous Epitopes*

Residues	Sequence	HLA-DR alleles						
		*0101	*0301	*0401	*0701	*0801	*1101	*1501
141–155	GNWQYFFPVIFSKAS	25	>100[‡]	7	0.1	3.2	0.6	3
146–160	FFPVIFSKASSSLQL	10	7	2	0.01	1.5	1.8	0.2
156–170	SSLQLVFGIELMEVD	7	90	45	0.03	7	28	0.18
171–185	PIGHLYIFATCLGLS	0.3	2.8	0.9	0.01	1.5	0.9	0.03
281–295	TSYVKVLHHMVKISG	15	26	70	0.02	0.01	0.03	0.5

The binding data are expressed in terms of relative binding capacity (IC_{50} μM), calculated as concentration of competitor peptide required to inhibit 50% of the binding of an allele-specific biotinylated peptide (indicator peptide).
[‡]IC_{50} values >100 μM are outside the sensitivity limits of the binding assay.

Intracytoplasmic staining for IL-4 and INF-γ expression, performed after $CD4^+$ T cell activation with PMA and ionomycin, revealed that 70% of the $CD4^+$ T cells produced INF-γ while no cells produced IL-4 (data not shown), suggesting that they belong mostly to the Th1 type.

To characterize the functional activity of the MAGE-3–specific $CD4^+$ T cells, we tested their killing potential against melanoma cells expressing the MAGE-3 protein and the HLA-DR molecules (Fig. 2 B). $CD4^+$ T cells showed cytolytic activity against OI TC and MD TC, which express the HLA-DR11 restricting allele, whereas they did not kill SK-Mel 28 and HT144, which express unrelated HLA-DR alleles (Fig. 2 A). To verify whether the cytolytic $CD4^+$ T cells recognized HLA-DR11–restricted MAGE-3 epitopes on melanoma cells, we first tested their lytic activity against HLA-$DR11^+$ LCL unpulsed, or pulsed with the synthetic peptides recognized in microproliferation assays. LCL pulsed with MAGE-$3_{281–295}$ were strongly recognized by the $CD4^+$ T cells, whereas no killing activity against LCL unpulsed or pulsed with MAGE-$3_{141–155}$ and MAGE-$3_{146–160}$ was detectable (Fig. 3 A). Second, we performed cold target inhibition experiments which showed that the lytic activity of $CD4^+$ T cells against OI TC was inhibited by the addition of LCL pulsed with MAGE-$3_{281–295}$ (Fig. 3 B), demonstrating that this sequence is indeed presented by HLA-DR11 on the OI TC melanoma cells. These results further demonstrate that MAGE-$3_{281–295}$ is naturally processed and forms a cytotoxic $CD4^+$ T cell epitope. Since the polyclonal $CD4^+$ T cells proliferated in the presence of the rMAGE-3 protein, and in addition to MAGE-$3_{281–295}$ they also recognized MAGE-$3_{141–155}$ and MAGE-$3_{146–160}$, we cannot exclude that these last two sequences may also yield natural epitopes, which are recognized by $CD4^+$ T cells with functional activity different from killing. Moreover, although $CD4^+$ T cells were mostly Th1 and had direct effector function upon tumor recognition, we cannot exclude that in vivo such $CD4^+$ T cells could also exert a helper activity in the induction phase of the immune response.

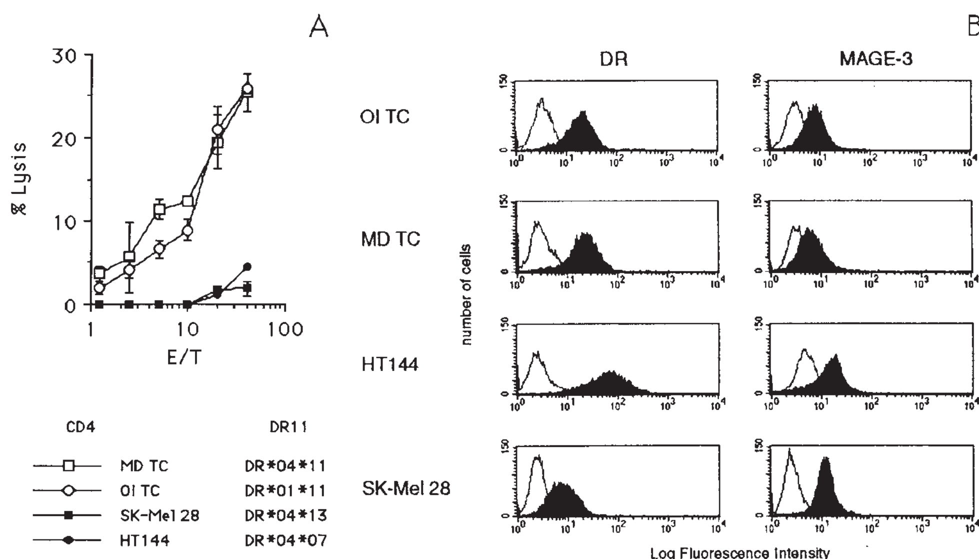

Figure 2. Cytolytic activity of MAGE-3–specific $CD4^+$ T cells. The data are representative of $n = x$ experiments, and are means of triplicate determinations ± SD. (A) Lytic activity against different HLA-DR–matched and unmatched melanoma cells ($n = 6$). HLA-DR types of $CD4^+$ T cells and melanomas are indicated at the bottom along with their symbols. (B) Cytofluorimetric analysis for HLA-DR (surface) and MAGE-3 (intracytoplasmic) expression in melanoma cells used as targets ($n = 4$). Filled histograms, stained sample; open histograms, background staining obtained with FITC-conjugated second-step reagent only.

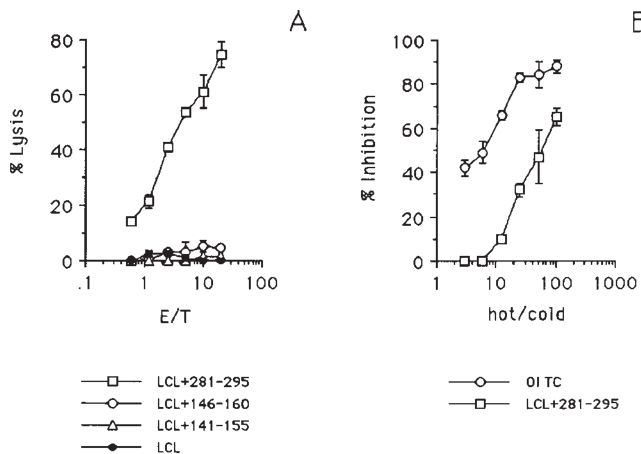

Figure 3. CD4+ T cells recognize MAGE-$3_{281–295}$ in association with HLA-DR11 on OI TC cells. The data are representative of $n = x$ experiments, and are means of triplicate determinations ± SD. (A) Lytic activity of CD4+ CTLs against LCL alone or LCL pulsed with MAGE-$3_{141–155}$, MAGE-$3_{146–160}$, and MAGE-$3_{281–295}$ ($n = 3$). (B) Cold target inhibition experiments ($n = 3$). Cold targets (OI TC [○] and LCL pulsed with MAGE-$3_{281–295}$ [□]) were used to inhibit the lytic activity of MAGE-3–specific CD4+ CTLs against hot OI TC (E/T ratio of 40:1). Percentage of specific lysis against OI TC cells in the absence of cold targets was 26 ± 1.2%.

One approach for identifying CD4+ T cell epitopes on a candidate protein is the use of overlapping synthetic peptides corresponding to the complete sequence of the protein. The major drawback of this approach is the number of peptide sequences that need to be tested, thus making this approach too expensive and time consuming. In this study, we used the TEPITOPE software package to computationally identify promiscuous HLA-DR binding sites starting from primary protein structures. We demonstrated that TEPITOPE predicted sequence segments capable of binding to multiple HLA-DR alleles. Furthermore, we showed that one or more of the predicted HLA-DR ligands were indeed naturally processed, thus confirming the validity of this approach. We expect that the application of TEPITOPE to other tumor-associated antigens will speed up identification of the antitumor CD4+ T cell epitope repertoire in humans.

Clinical trials based on the use of melanocyte-specific antigens (such as gp100, MART-1/Melan-A, and tyrosinase, for which CD4+ T cell epitopes were identified) are in progress in melanoma patients, and although no significant side effects were reported in a recent study that used a gp100 peptide for the treatment of HLA-A2+ patients (31), the development of autoimmune responses against normal tissue must be considered when using self-differentiation antigens as vaccines. The demonstration that MAGE-3 (i.e., an antigen not expressed in normal tissues, with the exception of testis and placenta, which are unlikely to be targets of T cells since they do not express MHC molecules), can form CD4+ T cell epitopes further supports its use for vaccination protocols in neoplastic patients using a mixture of synthetic peptides corresponding to CD8+ and CD4+ T cell epitopes.

Previous findings (13, 32, 33) reported a lytic activity of melanoma-specific CD4+ T cells. Here we give the molecular definition of an epitope able to stimulate cytolytic CD4+ T cells that can be grown in vitro with ease, raising the possibility of using those CD4+ T cells in protocols of adoptive transfer in neoplastic patients whose neoplasm expresses the MAGE-3 protein and the MHC class II molecules.

In conclusion, in this study we identified the first CD4+ T cell epitope on a tumor-specific antigen, and we verified that the approach used here to predict promiscuous CD4+ T cell epitopes yielded natural epitopes. It will be important to evaluate whether the identified CD4+ T cell epitopes are indeed promiscuous, making their use for peptide-based vaccines less allele dependent and more widely applicable.

We thank Giuseppe Consogno for excellent technical support and Catia Traversari for critical reading of the manuscript and helpful discussions.

This work was supported by the Fondazione Centro San Raffaele del Monte Tabor, the Italian Association for Cancer Research (AIRC), the Ministry of University and Scientific Research (MURST), and the Swiss National Fund.

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Received for publication 10 November 1998 and in revised form 15 December 1998.

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