[CANCER RESEARCH 62, 3630–3635, July 1, 2002]

Advances in Brief

Generation of NY-ESO-1-specific CD4+ and CD8+ T Cells by a Single Peptide with Dual MHC Class I and Class II Specificities: A New Strategy for Vaccine Design

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Abstract

The existence of overlapping CD8+ and CD4+ T-cell epitopes within certain tumor antigens provides an opportunity to test the hypothesis that relatively short peptides could be used to generate both CD8+ and CD4+ T cells against tumor. In this report, T-cell responses to a fragment of the tumor antigen NY-ESO-1 that contained an HLA-DP4-restricted helper T cell epitope as well as an HLA-A2-restricted cytotoxic T cell epitope were analyzed. One peptide, ESO:157–170 (SLLMWITQCFLPVF) was recognized by both NY-ESO-1-reactive CD8+ and CD4+ T-cell clones. Both CD4+ and CD8+ T cells were efficiently generated from the peripheral blood of multiple melanoma patients after *in vitro* stimulations using ESO:157–170. Dual-specific peptides containing both cytotoxic T-cell and helper T-cell epitopes may represent an attractive strategy of vaccine design aimed at generating tumor-reactive CD4+ and CD8+ T cells.

Introduction

Recently, therapeutic vaccines based on the genetic identification of MHC class I cancer antigens have been used in clinical trials (1). Many of the human trials have used immunization with recombinant viruses that encode cancer antigens, including adenovirus, vaccinia virus, and avipox virus; some involve the use of naked DNA or RNA as materials for immunization. T-cell responses are only sporadically generated by these immunization attempts (1–3). In contrast, peptide vaccines using MHC class I-restricted epitopes or epitope analogs from cancer antigens can readily lead to the generation of tumor-reactive CD8+ CTLs (4–7). Nevertheless, clinical responses are limited and do not apparently correlate with the presence of tumor-reactive CD8+ CTLs in the peripheral blood of patients (5, 7). One possible factor that may have limited the therapeutic impact of anti-tumor CTLs is the lack of CD4+ HTCs.[2] Evidence from animal models indicates that it may be necessary to engage both CD4+ and CD8+ T cells to develop more effective cancer vaccines (reviewed in Ref. 8). Recent advances in tumor antigen discovery have resulted in the identification of a number of CTL and HTC epitopes, some of which have been found in contiguous or overlapping regions within the primary amino acid sequence of these proteins. Previous studies demonstrate the presence of HLA-A2-restricted CTL epitopes, ESO: 157–165 and ESO:157–167 (both are recognized by the same T cells), as well as an overlapping HLA-DP4-restricted HTC epitope, ESO: 161–180, within the same region of NY-ESO-1 tumor antigen (9, 10). In this study, relatively short peptides containing overlapping CTL and HTC epitopes have been tested for their abilities to generate both CD4+ and CD8+ T cells against NY-ESO-1. A peptide with such ability may be an attractive candidate for the development of more effective cancer vaccines.

Materials and Methods

Patient PBMCs and Cell Lines. All of the PBMCs used in this study were from metastatic melanoma patients treated at the Surgery Branch, National Cancer Institute, NIH, Bethesda, MD. All of the melanoma lines and EBV B lines were generated at the Surgery Branch and maintained in RPMI 1640 (Life Technologies, Inc. Rockville, MD) supplemented with 10% FCS (Biofluid, Inc., Gaithersburg, MD). All of the melanoma lines used in Table 1 were described before (9). Lines 586mel and 1362mel were MHC class II positive without IFN-γ treatment, whereas 526mel was negative for MHC class II expression under the same conditions. F026mel was an early tumor culture generated from fine needle aspiration and showed low MHC class II expression in the absence of IFN-γ treatment. The cosA2 cell line was a stable cos cell line transfected with a plasmid expressing HLA-A2. 293CIITA was generated by transduction of the 293 cells with a retrovirus encoding the class II transactivator gene (CIITA); the resultant cell line was naturally HLA-A2 negative and expressed HLA-DP4 (9). Culture medium for T lymphocytes was RPMI 1640 with 0.05 mM β-mercaptoethanol, 300 IU/ml IL-2 (Chiron Corp., Emerville, CA), plus 10% human male AB serum provided by BioCheMed Inc. (Winchester, VA). The HLA-DP4-restricted CD4+ T-cell CT4–1 clone 12C was derived from a bulk line generated by ESO:161–180 (9). The HLA-A2-restricted CD8+ T-cell TE8–1 clone 8F was derived from a bulk line generated by *in vitro* stimulation using ESO:167–165,9V peptide variant (11). This T-cell clone recognized both ESO:157–165 and ESO:157–167 peptides.

Peptide Synthesis. Synthetic peptides used in this study were made using a solid phase method on a peptide synthesizer (Gilson Co. Inc., Worthington, OH) at the Surgery Branch of the National Cancer Institute. The quality of each peptide was evaluated by mass spectrometry (Biosynthesis Inc., Lewisville, TX).

***In Vitro* Sensitization Procedure, T-Cell Purification, and Cytokine Release Assays.** *In vitro* sensitization procedures using PBMCs as APCs were as described before (12). Briefly, peptides at a final concentration of 0.5 and 10 μM for MHC class I and class II epitopes, respectively, were used to stimulate 2.5×10^5 cryo-preserved human PBMCs in each well of a 96-well plate. Rapid expansion and cloning of human T cells were performed as described (12), and weekly restimulation was performed once or twice. IL-2 at a final concentration of 120 IU/ml was not added until day 8, 1 day after the second stimulation, and fresh IL-2 was added every 3 days thereafter.

The CD4+ and CD8+ T cells were purified from cell cultures using a negative selection approach, which negatively depleted CD8+ (or CD4+) T cells together with natural killer cells, B cells, monocytes, and granulocytes with magnetic beads (Dynal Biotech).

For specificity assays, cells were incubated with several targets, and supernatants were assayed for cytokine release. In brief, peptides at a final concentration of 10 μM for MHC class II epitopes and 1 μM for MHC class I epitopes were pulsed onto target cells. Unless specified, cells were incubated in RPMI

Received 4/1/02; accepted 5/17/02.

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[2] The abbreviations used are: HTC, helper T cell; EBV B, Epstein-Barr Virus infected B lymphocyte; PBMC, peripheral blood mononuclear cell; APC, antigen-presenting cell; ESO, NY-ESO-1; IL, interleukin; GM-CSF, granulocyte macrophage colony-stimulating factor.

Table 1 *CD4+ and CD8+ T-cell activities from reactive cultures of individual patient*

Patient	Peptide used for stimulation	IFN-γ secretion (pg/ml) against the following targets: Medium	cosA2/ESO: 91–110	cosA2/ESO: 157–167	293CIITA/ESO: 91–110	293CIITA/ESO: 161–180	526mel (A2+, DP4−, ESO−)	586mel (A2−, DP4+, ESO+)	1362mel (A2−, DP4−, ESO+)	F026mel (A2+, DP4^{low}, ESO+)
BL	p157–167	150	678	**>1500**[a]	199	252	565	164	178	**1121**
	p157–170	98	267	**1234**	112	**599**	61	**1276**	490	99
	p161–180	0	107	150	0	**>1500**	0	**287**	0	0
CJ	p157–167	No peptide reactive cultures were observed								
	p157–170	57	102	90	78	**844**	8	**536**	227	46
	p161–180	0	nd[b]	nd	12	**>1500**	0	0	0	0
CT	p157–167	0	361	**1358**	165	**1224**	102	**315**	106	**1137**
	p157–170	nd	30	**270**	30	**470**	20	**140**	30	**200**
	p161–180	326	nd	nd	219	**950**	88	111	124	36
FJ	p157–167	No peptide reactive cultures were observed								
	p157–170	755	177	111	131	**524**	0	**131**	41	**104**
	p161–180	59	nd	nd	42	**130**	0	**315**	121	89
TE[c]	p157–167	nd	140	**>1000**	nd	nd	nd	nd	nd	nd
	p157–170	nd	nd	nd	250	**1000**	nd	nd	nd	nd
	p161–180	nd	nd	nd	275	**850**	nd	nd	nd	nd

[a] Numbers in bold indicated cultures showing >100 pg/ml cytokine release and at least 2-fold above the background.
[b] Nd, not determined.
[c] Recognition of tumor cells by CD4+ and CD8+ T cells generated from TE has been shown previously (9). T cells generated in this study were only assayed for peptide reactivity.

1640 supplemented with 10% FCS for 90 minutes at 37 degree, followed by extensive wash. When serum-free medium was used, β2-microglobulin (Sigma-Aldrich, St. Louis, MO) was added to a final concentration of 1 μg/ml. Approximately 3×10^4 target cells were incubated with the same number of T cells overnight, and cytokine release was measured using GM-CSF ELISA kits (R&D Systems, Minneapolis, MN) or IFN-γ kits (Endogen, Inc., Woburn, MA).

HLA-A*0201 Peptide Binding Assays. Quantitative assays to measure the binding of peptides to soluble HLA-A*0201 molecules were based on the inhibition of binding of a radiolabeled standard peptide. These assays were performed as described previously (13).

Results

ESO:157–170 Is Recognized by Both CD4+ and CD8+ T Cells. The tumor antigen NY-ESO-1 belongs to the category of cancer/testis antigens that is widely expressed in a number of cancers as well as in normal testis (14). These features make NY-ESO-1 an attractive target for vaccine development against a number of epithelial cancers. The presence of overlapping CTL and HTC epitopes between amino acid residue 157 and 180 raised the possibility that a single peptide within this region of the NY-ESO-1 protein might be recognized by both MHC class I and class II-restricted T cells (Fig. 1*A*).

To examine this issue, a number of candidate peptides were tested for their ability to stimulate HLA-DP4-restricted CD4+ and HLA-A2-restricted CD8+ T-cell clones, which were generated using peptide ESO:161–180 and ESO:157–165,9V, respectively (described in "Materials and Methods"). Five peptides that differed in their COOH-terminal residues were pulsed on L023 EBV B (HLA-A2+ and HLA-DP4-) or 293CIITA (HLA-A2- and HLA-DP4+) cells. The peptide-pulsed cells were then used as targets for recognition by the CD8+ and CD4+ T-cell clones. As shown in Fig. 1*B*, CD4+ T-cell clone CT4–1-12C secreted significant amount of cytokines on stimulation with targets pulsed with the ESO:157–169, ESO:157–170, and ESO:157–171 peptides. However, the CD4+ T-cell clone poorly recognized targets pulsed with the ESO:157–167 and ESO:157–168 peptides. The CD8+ CTL clone TE8–1-8F recognized targets pulsed with ESO:157–168, ESO:157–169, ESO:157–170 and ESO:157–171 as well as ESO:157–165, and ESO:157–167 peptides (Fig. 1*C*). Recognition of longer peptides apparently was comparable with that of ESO:157–167 in this as well as additional experiments using CD8+ T-cell clones generated from a second melanoma patient (not shown). Among the longer peptides recognized by both CD4+ and CD8+ T cells, a 14-mer peptide ESO:157–170 was chosen for additional studies. A dose titration was then carried out to evaluate responses of CD8+ and CD4+ T-cell clones to ESO:157–170. As shown in Fig. 1*D*, target cells pulsed with ESO:157–170 could stimulate cytokine release from CD8+ and CD4+ effector T cells at a minimum concentration of ~0.03 and 0.3 μg/ml (0.02 and 0.2 μM), respectively.

ESO:157–170 Is Capable of Generating Both CD4+ and CD8+ T Cells *in Vitro*. *In vitro* immunogenicity of the gp100:209–217 peptide had been used previously to provide a rationale for the development of more effective clinical protocols (15). The ability of a single ESO:157–170 peptide to generate tumor-reactive CD4+ and CD8+ T cells *in vitro* was then examined. Two additional peptides, the HLA-DP4 epitope ESO:161–180, which appeared to stimulate only CD4+ T cells, and the HLA-A2 epitope ESO:157–167, which had been shown to significantly stimulated CD8+ T cells (11, 16), were also used to carry out *in vitro* stimulations.

In two previous studies, it had been suggested that patients with anti-NY-ESO-1 antibodies were more likely to have pre-existing HLA-DP4 CD4+ and HLA-A2-restricted CD8+ T cells (9, 17). Therefore, PBMCs from five melanoma patients with high-titer antibodies against NY-ESO-1 (data not shown) and who also expressed HLA-A*0201 and HLA-DPB1*0401/0402 were chosen for *in vitro* peptide stimulations.

To facilitate the generation of T cells by peptide *in vitro*, *in vitro* stimulations were carried out using a microculture technique described previously (18). Cell cultures were stimulated weekly with peptide-pulsed autologous PBMCs. The cellular immune responses against both the HLA-A2-epitope ESO:157–167 and the HLA-DP4-epitope ESO:161–180 were monitored after the third *in vitro* stimulation. Micro-well cultures were considered to be positive if they secreted >100 pg/ml of IFN-γ against cells pulsed with target peptides and at least twice the background response against cells pulsed with a control peptide. Cells reactive with the peptide epitopes were additionally analyzed for activities against transfected cells and tumor lines.

The IFN-γ responses of cultures with the strongest reactivity from each patient under each stimulation condition are shown in Table 1. All five of the patients developed T cells reactive with the DP4-restricted CD4+ T-cell epitope ESO:161–180 after three rounds of stimulation with the ESO:157–170 peptide. Two patients, BL and CT, developed T cells that directly recognized tumor lines expressing NY-ESO-1 and HLA-DP4. These T cells maintained their activity against the peptide epitope after depletion of the CD8+ population,

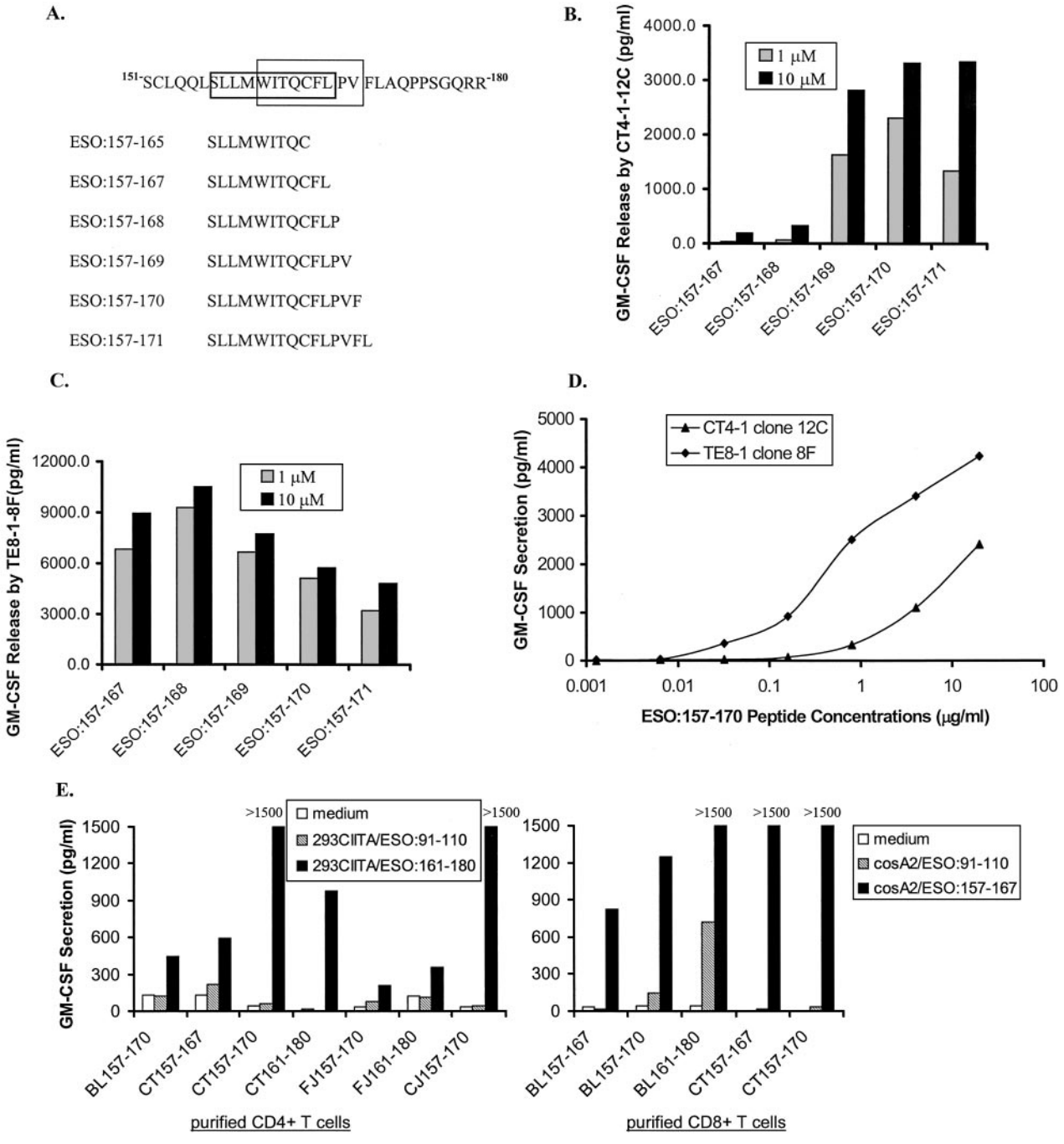

Fig. 1. *A*, the NY-ESO-1 tumor antigen contained overlapping CTL and HTC epitopes. The *smaller box* indicates the HLA-A2-restricted CTL epitope and the *larger box* indicates the core region of the HLA-DP4-restricted HTC epitope (9). Peptides used in this study are also listed. *B*, peptides of various lengths recognized by CD4+ CT4–1 clone 12C. *C*, peptides of various lengths recognized by CD8+ TE8–1 clone 8F. *D*, ESO:157–170 peptide pulsed on 1088 EBV B cells (A2+, DP4+) at different concentrations were recognized by both CD4+ and CD8+ T cell clones. *E*, activities of selected cell cultures after CD4+ or CD8+ T-cell purification. T-cell cultures as listed in Table 1 were purified using a negative selection beads ("Materials and Methods"), then incubated with the indicated targets. Explanation of legend: *e.g.*, BL157–167, cultures from patient BL generated with ESO:157–167 peptide.

demonstrating that the reactivity was mediated by CD4+ T cells (Fig. 1*E*). Two of the five patients, BL and CT, stimulated with the ESO:157–170 peptide appeared to contain T cells reactive to the HLA-A2-restricted peptide ESO:157–167. Similarly, these T cells maintained their reactivity after depletion of CD4+ T cells (Fig. 1*E*). Moreover, T cells raised from one of the two patients, CT, could directly recognize tumor cells expressing NY-ESO-1 and HLA-A2. CD4+ and CD8+ T-cell clones were generated from selected cultures and were shown to be able to recognize the NY-ESO-1 HLA-DP4 and HLA-A2 epitope, respectively (data not shown). Peptide dose titration experiment was carried out on CD8+ T-cell clones from patient TE, no apparent difference on the avidity of T cells generated by ESO: 157–167 and ESO:157–170 was observed (data not shown). These data indicated that *in vitro* stimulation with the ESO:157–170 peptide could generate NY-ESO-1-specific CD4+ and CD8+ T cells from multiple melanoma patients.

After the *in vitro* stimulation with ESO:157–167 peptide, CD8+ CTLs were generated from three of the five patients, BL, CT, and TE; CD4+ T cells were generated from one of the five patients. Stimulation with the peptide ESO:161–180 generated CD4+ T cells but not

Table 2 *Comparison of in vitro immunogenicity to generate CD4+ and CD8+ T cells by NY-ESO-1 peptides*

Patient	Number of *in vitro* stimulation	No. of wells of 24 wells recognizing A2 epitope			No. of wells of 24 wells recognizing DP4 epitope		
		Peptide used for *in vitro* stimulation			Peptide used for *in vitro* stimulation		
		ESO:157–167	ESO:161–180	ESO:157–170	ESO:157–167	ESO:161–180	ESO:157–170
BL	3	4 (2[a])	1 (1)	6 (6)	4 (2)	2 (1)	6 (6)
CJ	2	0	0	1	0	2	2
CT	2	1	0	1 (1)	8	11	19 (1)
FJ	3	0	0	0	0	1	1
TE	2	1	0	0	0	1	1

[a] Numbers in parenthesis indicated the number of cultures with both CD4+ and CD8+ T-cell activities.

NY-ESO-1-reactive CD8+ T cells from five patients who were evaluated.

Comparison of the *in Vitro* Immunogenicity of Different Peptides. To obtain a measurement for the relative immunogenicity of individual peptides, *i.e.,* ESO:157–167, ESO:157–170, and ESO:161–180, the number of individual microculture wells with reactivities against the peptide epitopes was determined after two to three weekly stimulations. MHC class I activity was tested using L023 EBV B cells (HLA-A2+ and HLA-DP4−), and MHC class II activity was tested using 293CIITA (HLA-A2− and HLA-DP4+) cells. The HLA-A2 peptide used for testing was ESO:157–165, which was different from the three peptides used for *in vitro* stimulation to avoid potential reactivity against a contaminant within the peptide preparation. For the same reason, ESO:160–174 instead of ESO:161–180 was used for testing the DP4-restricted CD4+ T-cell activity. As shown in Table 2, peptide-reactive CD8+ T cells were generated from three of five patients in a total of 8 microcultures when using the ESO:157–170 peptide. Peptide-reactive CD4+ T cells were observed from all five of the patients in a total of 29 microcultures after stimulation with the ESO:157–170 peptide. In contrast, ESO:161–180 peptide primarily raised peptide-reactive CD4+ T cells from five patients in a total of 17 microcultures, whereas CD8+ T-cell activity was observed only in one well after stimulation with the ESO:161–180 peptide. A total of six wells derived from three patients developed peptide-reactive CD8+ T cells after stimulation with the ESO:157–167 peptide; a total of 12 microcultures derived from two patients developed peptide-reactive CD4+ T cells. Therefore, ESO:157–170 appeared to be efficient at sensitizing both CD4+ and CD8+ T cells. It was also noteworthy that several of the microcultures from BL and CT generated using the ESO:157–170 peptide appeared to contain both CD4+ and CD8+ T-cell activities to NY-ESO-1.

ESO:157–170 May Directly Bind to HLA-A2 Molecule. Normally, MHC class I-binding peptides are 8–11 amino acids in length. However, in this study, peptides ranging between 9 and 15 amino acids in length were found to stimulate MHC class I-restricted CTLs (Fig. 1*C*). One hypothesis that could explain this apparent discrepancy was that cleavage of these longer peptides by proteases or peptidases in human serum might generate peptides of the appropriate length to allow the binding of MHC class I molecules. An attempt was then made to identify the optimal peptides by measuring the HLA-A∗0201 binding affinity of peptides of different lengths within the region of amino acid 157–170. As shown in Table 3, the HLA-A2 epitopes reported previously, ESO:157–165 and ESO:157–167, appeared to bind with very low affinity to the HLA-A∗0201 molecule. In contrast, the apparent high binding capacity of 9- and 10-mer peptides beginning with amino acid residues 158 and 159 suggested that one of these two peptides might represent the epitope that was present on the cell surface and recognized by T cells that apparently recognized ESO:157–167.

To test the possibility that ESO:158–167 or ESO:159–167 were generated by the protease or peptidase activity present in human serum, peptides of various lengths were pulsed onto T2 cells in the presence and absence of human serum for 1 h at room temperature. Cells were extensively washed and then cocultured with effector CTL in medium containing serum. As shown in Table 3, TE8–1 clone 8F recognized peptides ESO:157–165, ESO:157–167, and ESO:157–170 to similar extent regardless of whether human serum was present in the medium or not during the peptide pulsing period. These peptides were also recognized to a similar extent by an ESO:157–167-specific CTL clone from another patient (data not shown). In addition, peptides that appeared to have high binding capacities, ESO:158–167, ESO:159–167, and ESO:161–169, were not recognized by an ESO:157–167-specific T-cell clone, which rules out the possibility that the real epitope was resulted from an NH_2-terminal truncation of the ESO:157–167 peptide. Moreover, CTL clones were also able to recognize the same peptides pulsed on untreated as well as fixed HLA-A2+ APC, which did not seem to support a hypothesis that peptides were processed and represented by the APC. Overall, these results did not indicate that peptide epitopes were generated by serum proteases. Therefore, longer peptides from the ESO amino acid 157–170 region might be capable of binding directly to HLA-A2.

Table 3 *HLA-A*0201 binding capacity by NY-ESO-1 peptides of different lengths and their recognition by CD8+ CTL*

Peptide	Position	IC_{50} (nM)	Recognition by TE8-1 clone 8F (IFN-γ secretion in pg/ml)					
			Experiment 1				Experiment 2	
			Without serum		10% Human serum		Without serum[a]	
			1 μg/ml	0.1 μg/ml	1 μg/ml	0.1 μg/ml	Fixed	Untreated
SLLMWITQC	p157–165	21,070	3,855	1,733	6,115	2,006	>5,000	>5,000
SLLMWITQCFL	p157–167	>50,000	1,303	1,326	9,110	3,054	>5,000	>5,000
SLLMWITQCFLPVF	p157–170	14,938	1,952	1,625	6,585	1,138	>5,000	>5,000
LLMWITQCFL	p158–167	43	0	0	249	3	0	0
LMWITQCFL	p159–167	78	0	0	0	0	0	0
WITQCFLPV	p161–169	173	0	0	0	0	0	0

[a] Peptides were pulsed onto Baldwin EBV B cells at 1 μg/ml. Untreated as well as paraformadehyde-fixed EBV B cells were used as targets. Untreated but not fixed cells were able to present a gp100 protein to relevant CD4+ T cells, indicating that the fixation was effective (data not shown).

Discussion

A number of studies in animal models have demonstrated a role for both CD8+ CTLs and CD4+ HTCs in tumor immunotherapy (reviewed in Refs. 8, 19). Because epitope-based peptide vaccines had been successfully used in clinical trials to raise antigen-specific CTLs in melanoma patients, an attempt was made to identify peptides that were capable of stimulating both CD4+ and CD8+ T cells.

A number of MHC class II-restricted epitopes partially overlapping with MHC class I-restricted epitopes had been described. For example, the HLA-DR53-restricted helper epitope gp100:175–189 from tumor antigen gp100 partially overlapped with an HLA-A2-restricted CTL epitope, gp100:177–186; the HLA-DR7-restricted helper epitope gp100:74–89 partially overlapped with two CTL epitopes, gp100:70–78 and gp100:87–95 (20). Extensions of a minimal epitope might be used to obtain a peptide that contained both class I and class II epitopes. The difficulty with this approach was that whereas the open ends of the class II binding cleft generally allow extensions, the more closed nature of the class I binding cleft generally interferes with the binding of peptides that are longer than the minimal epitope of 9–10 amino acid residues. Nevertheless, we found in this study that peptides ranging from 9 to 15 amino acids in length apparently stimulate HLA-A2 restricted CTLs. These peptides of various lengths required the same NH_2-terminal amino acid residue but allowed extension at the COOH termini. We speculated that the Pro near the COOH terminal might make a rigid turn of a longer peptide and allow it to fit the HLA-A2 peptide-binding groove. The only other case in which a 14-mer peptide worked as an MHC class I epitope was reported by Probst-Kepper *et al.* (21). However, in that case the peptide presumably formed a bulge in the middle as both the NH_2- and COOH-terminal residues were required for proper binding to the HLA-B35 molecule.

Previous studies provided evidence that some CTL response might be linked to HTC responses against an overlapping epitope within the same protein. For tumor antigen NY-ESO-1, previous reports indicated that CTL responses against ESO:157–167 was closely associated with the presence of antibodies against the protein (17). We also reported that the ability to generate CD4+ T cells reactive with the HLA-DP4-restricted ESO:161–180 epitope was associated with the presence of antibodies against NY-ESO-1 (9). This might be simply because CTLs and helper T-cell responses against these two closely situated peptides were associated. Results from this study also indicated that at least three of the five HLA-A2 and HLA-DP4 patients developed T-cell responses against both epitopes after *in vitro* stimulations (Table 2). Wang *et al.* (22) also reported in a malaria DNA vaccine clinical trial that patients developed concordant CTL and helper T-cell responses against overlapping MHC class I and HLA-DR epitopes from *Plasmodium falciparum* (22). Moreover, they reported that the most antigenic class I peptides were those overlapping with DR peptides in the normal volunteers tested. Therefore, peptides containing overlapping class I and class II epitopes might be important candidates for vaccine development.

Previously, vaccinations were tried using a mixture of CTL and helper T-cell epitopes, as well as peptides consisting of covalently linked CTL and helper epitopes (23, 24). Whereas these approaches might enhance the CTL induction compared with immunizations with CTL epitopes alone, we speculated that using natural peptides of dual MHC class I and class II specificities might be more advantageous. In theory, peptides composed of natural CTL and helper T-cell epitopes could be loaded onto the same APCs and bring both CTL and HTCs together. This allows the close contact of APCs, CD4+, and CD8+ T cells, and enhances the direct communications among these cells through means such as cross-linking molecules and cytokines. Some of these interactions have been demonstrated as critical for the initiation of an effective immune responses, for instance, the CD40-CD40L interaction between APCs and CD4+ T cells plays important roles in activation of dendritic cells by CD4+ T cells; and cytokines secreted by CD4+ T cells are important to activate and expand CD8+ CTLs (19). Natural peptides of dual MHC class I and class II specificities may also be advantageous compared with chimeric peptides formed by linkage of CTL and helper epitopes because the later may not be processed properly by APCs and may result in the presentation of artificial epitopes from the linkers. Furthermore, most long peptides consisting of covalently linked epitopes may not be presented by MHC class I molecules because of the lack of cross-presentation if they do not bind to MHC class I molecules directly.

It has been shown before that CTL clones with the same specificity generated by the same peptide may show heterogeneous functions. Some clones may secret high levels of cytokines and some clones may have higher lytic activity on stimulating with the same target (25). In another study, it was shown that GM-CSF and IFN-γ but not IL-2, tumor necrosis factor α, IL-4, or IL-10 may be better correlated with the lytic activity of peptide activated CTLs (26). Therefore, we used cytokine release assays (IFN-γ or GM-CSF) in this study to show CTL specificities without specifically addressing the lytic function of these CTLs.

In summary, for the first time a single peptide was demonstrated as capable of generating both CD4+ and CD8+ T cells against cancer. Thus, peptides with dual class I and class II specificities represented a new strategy for vaccine development.

Acknowledgments

We thank Dr. John Wunderlich, Jennifer Westwood, and Tom Shelton of the Surgery Branch Tumor Infiltrating Laboratory for providing clinical samples for this study. We also thank Dr. Maria Parkhurst and John Riley for synthesizing peptides used in this work.

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