Identification and Characterization of a T-Helper Peptide from Carcinoembryonic Antigen

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ABSTRACT

Purpose: The purpose of this research was to identify promiscuous T-helper cell determinants (THd) from carcinoembryonic antigen (CEA) to be used to prime T-cell help for cancer therapy. CEA was selected because this antigen is expressed in an important variety of carcinomas.

Experimental Design: Potential promiscuous THd from CEA were predicted using available computer algorithms. Predicted peptides were synthesized and tested in binding experiments to different HLA-DR molecules. Binder peptides were then used to prime T-cell responses both in vitro and in vivo.

Results: Twenty 15-mer peptides from CEA were predicted to bind to different HLA-DR molecules. The promiscuous character of these peptides was demonstrated in binding experiments. Fifteen of 20 peptides tested were able to bind to HLA-DR4, but only CEA (625–639) was shown to be presented after processing of recombinant CEA. CEA (625–639) was also found to be presented by HLA-DR53. Moreover, immunization of HLA-DR4 transgenic mice with CEA (625–639) in conjunction with class I epitope OVA (257–264), induced a CTL response specific of OVA.

Conclusions: CEA (625–639) might be a relevant promiscuous THd peptide for cancer therapy.

INTRODUCTION

T-cell immunotherapy is one of the most promising tools in cancer therapy. Different preclinical and clinical studies have demonstrated the ability of T lymphocytes to control tumor growth (1, 2). Because tumor-specific CTLs are responsible for the lysis of tumor cells, many research groups have channeled their efforts to the induction of CTL responses and to the identification of T-cytotoxic cell determinant peptides (TCds) from human tumor antigens (3–15). These TCds are presented at the surface of tumor cells in the form of a complex TCd-MHC-I, which is recognized by the T-cell receptor of CTL. However, despite the well-established role of CD4 T cells to induce immune responses, and in particular antitumor responses (16–20), much less effort has been put to identify T-helper determinants (THds) from tumor antigens.

Recognition of THd presented by MHC class II molecules on antigen-presenting cells (APCs) activates CD4 T-cells (HTLs). This activation is crucial for the generation and maintenance of other antitumor immune responses, such as CTLs, antibody producing B-cells, and natural killer cells. The pattern of cytokines liberated by the HTLs, as well as direct cell-to-cell contact through molecules such as CD40-CD40l, determine the fate of the response. Immunization experiments with peptide constructs (or peptide mixtures) containing THds and B-cell epitopes (21, 22) or TCds (23–25) have been very useful not only to understand the role of THds in antibody and CTL induction but to provide general guidelines for the use of synthetic peptides as components of a vaccine. Thus, characterization of THd in the sequence of tumor antigens is of paramount importance for the development of antitumor vaccines as well as the monitoring of T-cell responses in cancer patients.

Carcinoembryonic antigen (CEA) is a glycoprotein expressed by a high proportion of colorectal, gastric, and pancreatic carcinomas (26). Several studies have described different TCds in the sequence of CEA presented by HLA class I molecules (12, 13, 27–29). However, little has been done to identify THds from this antigen. In a previous publication (30) we reported a peptide from CEA presented by HLA-DR4, DR7, and DR9 molecules that was naturally processed and presented to HTLs. Due to the large variety of HLA class II molecules in humans, which are able to present different peptides, we decided to search for additional promiscuous THds within the sequence of CEA that might be useful in the therapy of a wide population of patients with cancer. To do this, we have combined the prediction of THd from CEA using available algorithms for different HLA-DR molecules together with binding assays of predicted peptides. Binder peptides were then used to induce CD4 T-cell responses, both in HLA transgenic mice and human models. We describe below the steps taken to achieve this goal.

MATERIALS AND METHODS

Antigens. Potential promiscuous HLA-DR-binding peptides were predicted from CEA using the algorithms for DRB1*0101, DRB1*0401, and DRB1*0701 described by Southwood et al. (31), as well as by algorithms from our...
laboratory (32). Peptides were synthesized manually in a multiple peptide synthesizer using Fmoc chemistry. Synthesis and biotinylation of peptide HA (306–320; APKYVKQNTLKLATG) from Influenza A/Texas/77 virus hemagglutinin was carried out as described previously (32). Recombinant CEA protein was purchased from Calbiochem (San Diego, CA). Recombinant hippocalcin-like 1 protein (Hpcal 1) was expressed in Escherichia coli as a glutathione S-transferase-fusion protein. The Hpcal 1 protein was concentrated, and the purity of the protein was verified by SDS-PAGE.

Mice. HLA-DR4 transgenic mice were obtained from Tac- onic (Germantown, NY). C57Bl6 mice were obtained from Harlan (Barcelona, Spain). They were maintained in pathogen-free conditions and treated according to guidelines of our institution.

Cell Lines. EBV-transformed B-lymphoblastoid cell lines HOM2, WT8, RSH, BOLETH, MOU, OLGA, and SWEIG, used in binding assays, were grown as described previously (32). EL-4 thymoma cells (H-2d) were used as target cells in chromium release assays with CTL from HLA-DR4 transgenic mice. Mouse fibroblast cell lines (t-cells) transfected and expressing individual human MHC class II molecules were kindly provided by Dr. Robert W. Karr (Pfizer Global R&D, New London, CT) and by Dr. Takehiko Sasazuki (Kyushu University, Fukuoka, Japan). The colon adenocarcinoma cell line SW403 and the Jurkat T-cell lymphoma were purchased from American Type Culture Collection (Manassas, VA). Cell line MNK45 was supplied by the Japanese Cancer Research Resources Bank (Tokyo, Japan).

Peptide Binding Assays. Binding assays were performed basically as described previously (32) but using as control the peptide from influenza hemagglutinin HA306–320 (APKYVKQNTLKLATG). We used this sequence instead of CPKYVKQNTLKLATG described previously (33) to avoid potential disulfide bridge formation via the NH2-terminal cysteine. The relative inhibitory capacity of peptides from CEA was calculated according to the formula:

Relative inhibitory capacity = 100 x (signal inhibition with peptide tested)/signal inhibition with unbiotinylated HA306–320.

Immunization. Groups of three HLA-DR4 transgenic mice were immunized s.c. in the foot pads and in the base of the tail with 4 μg/mouse of CEA or with 50 nm of T-helper peptide emulsified in incomplete Freund’s adjuvant. In immunization experiments for CTL induction, mice were immunized with 50 nm of CTL epitope OVA (257–264) with or without 50 nm of the T-helper peptide from CEA. Two weeks later, animals were sacrificed, and lymph nodes were removed, homogenized, and pooled for immunological analysis.

Mouse T-Cell Lines. T-cell lines were grown by periodic cycles of peptide stimulation and resting. Briefly, lymph node cells were pulsed for 2 h with peptide (10 μM), washed, and grown at 2 x 106 cells/ml in 24-well plates. Four days later, cells were washed and plated (4 x 105/well) with mitomycin-C-treated syngeneic spleen cells (4 x 106/well). One week later, T cells were cultured again with peptide-pulsed mitomycin-C-treated spleen cells for 4 days, and these cycles were repeated.

Stimulation of Cells for the Study of Mouse HTL Responses. Lymph node cells were plated at 8 x 105 cells/well in 0.2 ml in U-bottomed 96-well plates in the presence of peptides or recombinant CEA. Cells cultured in the absence of antigen were used as negative control. In the case of T-cell lines, effector cells were used at the end of the resting period. T cells (5 x 103) were cultured with mitomycin-C-treated syngeneic spleen cells (2 x 103) in the absence or in the presence of the different antigens. In both assays, supernatants were harvested 2 days later, and IFN-γ was measured by ELISA (BD-PharMingen), according to the manufacturer’s instructions. On day 3, cells were pulsed with 0.5 μCi/well of tritiated thymidine for 18 h, harvested, and thymidine incorporation was determined in a scintillation counter (Top-count; Packard, Meridan, CT).

Measurement of CTL Responses. Lymph node cells from animals immunized with peptides were incubated with CTL epitope OVA (257–264) (10 μM) for 2 h at 37°C, washed twice, and cultured in 24-well plates at 7.5 x 106 cells/well. Two days later, IL-2 (2.5 units/ml) was added to the wells, and on days 6–7, cells were harvested for chromium release assays using peptide-pulsed EL-4 cells as targets.

In Vitro Induction of Human Antigen-Specific HTL Lines with Synthetic Peptide. The procedure selected for the generation of tumor antigen-reactive HTL lines using peptide-stimulated peripheral blood mononuclear cells (PBMC) was carried out as described (30, 34, 35). The Institutional Review Board on Human Subjects (Mayo Foundation) approved this research, and informed consent for blood donation was obtained from all of the volunteers.

Measurement of Antigen-Specific Responses with HTL Lines. CD4+ T cells (3 x 105/well) were mixed with irradiated APCs in the presence of various concentrations of antigen (peptides, recombinant protein, or tumor lysates), in 96-well culture plates. APCs consisted of PBMCs (1 x 105/well), HLA-DR-expressing t-cells (3 x 105/well), or dendritic cells (DC; 5 x 105/well). Tumor cell lysates were prepared by three freeze-thaw cycles of 1 x 106 tumor cells, resuspended in 1 ml of serum-free RPMI 1640. Lysates were used as source of antigen at 5 x 105 cell equivalents/ml. Culture supernatant were collected after 48 h for measuring antigen-induced lymphokine (granulocyte macrophage colony-stimulating factor) production by the HTL using ELISA kits (PharMingen, San Diego, CA). To determine MHC restriction molecules involved in antigen presentation, blocking of the antigen-induced response was investigated by adding anti-HLA-DR monoclonal antibody L243 (IgG2a, prepared from supernatants of the hybridoma HB-55 obtained from the American Type Culture Collection) or anti–HLA-A, -B, -C monoclonal antibody W6/32 (IgG2a; American Type Culture Collection). All of the antibodies were used at a final concentration of 10 μg/ml throughout the 48-h incubation-period. All of the assessments were carried out at least in triplicate and results correspond to the mean values with the SD of the mean.

RESULTS
Identification of Peptides with Ability to Bind to Different HLA-DR Molecules. To identify promiscuous Thd of potential use in cancer therapy, we selected peptides from the sequence of CEA that were predicted as promiscuous binders, according to algorithms described by Southwood et al. (31) as well as by our laboratory (32). Predicted peptides (shown in
Table 1) were synthesized and tested in binding assays. It was found that 15 of the 20 predicted peptides (75%) were able to bind to at least 3 of the 7 HLA-DR alleles tested (43% or more). HLA-DR1 and HLA-DR4 were the molecules to which a higher proportion of peptides were able to bind, 15 of 20 (75%) in both cases (Fig. 1).

Induction of T-Cell Responses in HLA-DR4 Transgenic Mice by Using Peptides Derived from CEA. Because peptides able to bind to HLA-DR molecules might not be generated after antigen processing (cryptic THd), to identify relevant THd to be used in therapy it is necessary to investigate if processing leads to adequate peptide presentation. To address this point we chose HLA-DR4 allele as the starting point, and immunized HLA-DR4 transgenic mice with recombinant CEA. Lymph node cells from these animals were stimulated with CEA-derived peptides to measure cell proliferation. Five peptides (indicated with an asterisk) as well as CEA induced a marginally higher cell proliferation with respect to cells not stimulated with antigen (Fig. 2). These differences did not provide a clear answer on whether the peptides were presented after antigen processing, and recognized by CEA-immune splenocytes. Thus, we immunized HLA-DR4 mice with these five peptides and studied the responses induced. Only peptides CEA (199–213), CEA (625–639), and CEA (665–679) were found to be immunogenic. However, although cells from these immunized mice were able to recognize the peptides (Fig. 3), these responses were not of a high magnitude, and they were not able to recognize CEA protein (data not shown). We also immunized mice with T-helper peptide CEA (653–667) identified previously by us (30) and found that it was also able to induce similar proliferative responses (data not shown). We hypothesized that in some cases, this lack of response against CEA could be due to the low frequency of antigen-specific cells, and that enrichment of these antigen-specific cells in the population would allow us to discriminate between responses against peptides generated after natural processing and responses against cryptic epitopes, that is, peptide-specific. For this reason, we decided to grow T-cell lines specific of these three peptides by repeated in vitro stimulation of cells with the corresponding peptide. Despite many attempts, only T-cell lines specific of CEA (625–639) and of CEA (199–213) could be obtained. Both cell lines were tested in proliferation assays as well as in IFN-γ production after stimulation with peptide or with recombinant CEA. Both cell lines recognized the corresponding peptide, but only the cell line specific for CEA (625–639) was able to recognize recombinant CEA (Fig. 4, A–D). Moreover, experiments using different APCs showed that only those cells bearing HLA-DR4

![Fig. 1](image-url)
were able to stimulate CEA (625–639)-specific T cells (Fig. 4E). These results show that peptide CEA (625–639) but not CEA (199–213) is naturally generated after processing of CEA and presented by HLA-DR4.

**Efficacy of CEA (625–639) Derived T-Cell Help on the Induction of CTL Responses.** THds have been successfully used in combination with TCds in immunization experiments due to the role of HTLs on the induction of CTL responses. To get insight on the efficacy of CEA (625–639) to provide T-cell help for CTL induction, we immunized HLA-DR4 transgenic mice with peptide OVA (257–264), a well-characterized TCd from ovalbumin (36), in the presence or absence of THd peptide CEA (625–639). Only coimmunization of CEA (625–639) and OVA (257–264) was able to induce a CTL response (Fig. 5), showing that CEA (625–639) is required for CTL induction and suggesting that this THd might be of relevance in human cancer therapy.

**Induction of CEA (625–639) Specific T-Cell Responses by Using Human Cells.** To confirm results obtained in transgenic mice and to identify other possible HLA-class II molecules that could also present peptide CEA (625–639) we decided to study its THd character in humans. Thus, a HTL clone was selected from an HLA-DR4/9, -DR53 normal individual by repeated stimulation of CD4+/H11001 T cells with peptide and APC as described previously (30). The T-cell response to CEA (625–639) was inhibited with anti-DR but not with anti-class I antibodies, both when CEA (625–639) was presented by PBMC or by DC (Fig. 6A). Also, when mouse fibroblasts transfected with human HLA-DR genes were used as APCs, it could be demonstrated that the HTL clone recognized CEA (625–639) in the context of HLA-DR4 and HLA-DR53, but not in the context of HLA-DR9 (Fig. 6B). Moreover, when we tested the efficacy of PBMCs and DCs to present the peptide, it was found that DCs required ~100 times less CEA (625–639) than PBMCs to attain the same response, expressed in pg/ml of granulocyte macrophage colony-stimulating factor (Fig. 6C).

Generation of CEA (625–639) after natural processing of CEA in the human system was also studied. These experiments showed that the T-cell clone specific for CEA (625–639) could be stimulated with DC and CEA but not with DC and Hpcal I (a protein not related to CEA), eliciting the production of granulocyte macrophage colony-stimulating factor, and demonstrating again that CEA (625–639) is naturally processed and presented (Fig. 7A). Moreover, when
this clone was stimulated in vitro with lysates from different cell lines, only those lysates obtained from CEA-expressing lines (SW403 and MKN45) but not those from CEA-negative lines (Jurkat) were able to stimulate the T cells. Moreover, and similarly to results against peptide pulsed APC (Fig. 6A), T-cell response against tumor lysates from CEA-expressing lines could only be inhibited by anti-DR but not anti-class I antibodies, confirming that the peptide generated after natural processing is also presented by these class II molecules (Fig. 7B).

**DISCUSSION**

Development of vaccines against tumor-associated antigens is one of the main goals of tumor immunology. Most studies aimed at inducing strong antitumor cellular immune responses have been directed to prime CTL responses. Accordingly, a great effort has been done to characterize this response in cancer patients, and different tumor antigens and CTL epitopes within these sequences have been identified. However, there is growing evidence of the importance of CD4 T cells in the development of efficient antitumor immune responses. Together with their known role on the production of cytokines that may help the induction of antibodies and CTL responses (37), CD4 T cells activate DCs for efficient antigen presentation (38–40). Moreover, a direct effect of CD4 T cells on tumor

*Fig. 4 Recognition of antigens (peptides or recombinant carcinoembryonic antigen; CEA) by T-cell lines specific of peptides CEA (625–639) and CEA (199–213). HLA-DR4 transgenic mice were immunized s.c. with peptide CEA (625–639) or CEA (199–213) emulsified in incomplete Freund’s adjuvant and T-cell lines were grown after several rounds of stimulation with the corresponding peptide. T cells specific for CEA (625–639) (closed symbols) or CEA (199–213) (open symbols) were stimulated with the corresponding peptide (A and B) or with recombinant CEA (C and D) in the presence of syngeneic HLA-DR4 spleen cells. Cell proliferation (A and C) and IFN-γ production (B and D) was measured in each case. E, T cells specific for CEA (625–639) were stimulated with or without peptide (10 μM) in the presence of different antigen-presenting cells and cell proliferation was measured; bars, ±SD. rCEA, recombinant CEA.*

*Fig. 5 Induction of CTL responses by immunization with helper peptide carcinoembryonic antigen (CEA) (625–639) and OVA (257–264) CTL epitope. HLA-DR4 transgenic mice were immunized s.c. with CTL epitope OVA (257–264) with or without CEA (625–639). Two weeks later, animals were sacrificed and lymph node cells were stimulated with OVA (257–264). A, IFN-γ production in the supernatants was measured 2 days later. B, lytic activity of in vitro stimulated lymph node cells from animals immunized with OVA (257–264) (open symbols) or with OVA (257–264) plus CEA (625–639) (closed symbols) was measured against EL-4 target cells incubated with or without OVA (257–264). Data represent the Δ specific lysis, which was calculated by subtracting the percent lysis with unpulsed target cells from the percent lysis with peptide-pulsed cells; bars, ±SD.*
Fig. 6 Human HTL response to peptide carcinoembryonic antigen (CEA) (625–639). HTL clone was selected from an HLA-DR4/9, -DR53 normal individual by weekly stimulation of CD4 T cells with peptide as described in “Materials and Methods.” A, autologous peripheral blood mononuclear cells (PBMC) or dendritic cells (DC) were used as antigen-presenting cells (APC), to measure the T-cell response to peptide CEA (625–639) in the presence and absence of anti-HLA-DR monoclonal antibody, L243 at 10 μg/ml, anti-HLA class I monoclonal antibody, W6/32 at 10 μg/ml. B, mouse L-cell fibroblasts transfected with various human HLA-DR alleles were used as APCs to present peptide CEA (625–639) (3 μg/ml). Values shown are the means of triplicate determinations; bars, ±SD. Columns without error bars have SDs too small to appear in the figure. C, dose-response curve of the human HTL clone for peptide CEA (625–639). Autologous PBMC (□) or DC (○) were used as APCs. Each data point represents the mean of triplicate samples where the SD values were consistently <10% of the mean (data not shown). GM-CSF, granulocyte macrophage colony-stimulating factor.

growth through the production of cytokines such as IFN-γ has also been described (20). Maintenance of antitumor memory CTL response is another important role of CD4 T cells. In this case, it has been described recently that for a proper memory CTL activity, antigens recognized by CTL and CD4 T cells should belong to the same antigen (41). Thus, in the recent years, several groups have studied CD4 T-cell responses against tumor antigens known previously to be recognized by CTL (34, 35, 42–46). Characterization of CD4 T-cell epitopes within these antigens is of crucial importance in the design of a vaccine. The use of such peptides would provide sufficient amount of well-characterized naturally processed immunogens. We have characterized several CTL epitopes in the sequence of CEA, presented by different HLA class I molecules (12, 13, 28, 29); however, to our knowledge, there is only a CD4 epitope in CEA that we described recently (30). Thus, we decided to search for new epitopes that could be useful for therapy against CEA expressing tumors.

For this purpose we used a strategy combining HLA-DR binding prediction algorithms, HLA-DR binding assays, and T-helper induction experiments in the HLA-DR4 transgenic mouse model and in humans. Such an approach allowed us to initially screen 20 peptides predicted as binders to different HLA-DR molecules, which was confirmed by binding assays. Because antigen natural processing and presentation is a requisite for a peptide to be used in therapy, the HLA-DR4 transgenic mice were used as a model of T-cell induction. HLA-DR4 is an allele presented by ∼25% of the population, making it very useful as a starting point for epitope characterization. Indeed, several studies have used these animals as initial screening for CD4 epitopes in tumor antigens, and epitopes characterized in this model have also been confirmed using human cells (43, 47, 48). Among the 15 peptides able to bind to HLA-DR4, only CEA (625–639) was naturally processed and presented. CEA (199–213) induced a peptide-specific response that was unable to recognize CEA. Lack of generation of this peptide after natural processing may account for this unresponsiveness against CEA, or alternatively, a lower affinity of the T cells recognizing CEA (199–213) compared with those recognizing CEA (625–639), as shown in Fig. 4, A and B. Regarding the other immunogenic peptide, CEA (665–679), because we were unable to grow lines specific for this peptide we could not study processing and presentation. Thus, our results do not exclude the possibility that it might be generated and presented by HLA-DR4. Thus, although binding algorithms narrow the number of potentially useful peptides, T-cell studies were needed to identify the naturally processed and presented peptide.

Because binding experiments had shown that CEA (625–639) could bind to DR4, as well as to DR1 and DR8, it was interesting to study this peptide in human donors presenting different HLA molecules. By using periodic stimulation of T cells with peptide-pulsed APCs, a T-cell clone specific for this peptide was grown. It was found that CEA (625–639) was presented not only by DR4, but also by DR53, reinforcing the promiscuous character of the peptide. As in the mouse system, CEA (625–639) is also naturally processed and presented by human cells. These results are very relevant, because HLA-DR53 is an allele with a prevalence of ∼50% of the Caucasian population, making CEA (625–639) potentially useful in cancer
Characterization of a T-Helper Peptide from CEA

By DR4 with presentation of OVA (257–639) transgenic mice, combining presentation of CEA (625–639) and from CEA-negative T-cell lymphoma line Jurkat were tested for their capacity to stimulate the HTL clone. The ability of anti-HLA-DR monoclonal antibody L243 (10 μg/ml) and anti-HLA class I monoclonal antibody W6/32 (10 μg/ml) to inhibit the recognition of tumor lysates presented by autologous DCs was also assessed. Values shown are the triplicate determinations; bars, ± SD. Columns and symbols without error bars had SD <10% the value of the mean. GM-CSF, granulocyte macrophage colony-stimulating factor.

In conclusion, a new promiscuous THd from CEA has been characterized, which is naturally processed and presented by different HLA class II molecules. Due to the importance of CD4 T-helper cells in the development of antitumor immune responses, CEA (625–639) should be considered as a candidate to be included in therapies aimed at inducing responses against CEA-expressing tumors.

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