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ABSTRACT

Purpose: **The product of the carcinoembryonic antigen (CEA) gene is an attractive candidate for T-cell-based immunotherapy because it is frequently expressed in epithelial solid carcinomas. Although many CEA peptide epitopes capable of stimulating CTLs have been identified, no MHC class II-restricted T helper epitope has yet been reported.**

Experimental Design: **The amino acid sequence of CEA was examined for the presence of potential T helper epitopes, and candidate peptides were used to stimulate *in vitro* T-cell responses.**

Results: **We describe here that using an algorithm to identify promiscuous helper T-cell epitopes, a peptide of CEA occupying residue positions 653 to 667 ($CEA_{653–667}$), was effective in inducing *in vitro* T helper responses in the context of the HLA-DR4, HLA-DR7, and HLA-DR9 alleles. Most significantly, some of the peptide-reactive helper T lymphocytes were also capable of recognizing naturally processed antigen in the form of recombinant CEA protein or cell lysates from tumors that express CEA. Interestingly, the newly identified helper T-cell epitope was found to overlap with a previously described HLA-A24-restricted CTL epitope, $CEA_{652–660}$, which could facilitate the development of a therapeutic vaccine capable of eliciting both CTL and T helper responses in patients suffering from epithelial carcinomas.**

Conclusion: **These results indicate that T helper lymphocytes are capable of recognizing CEA as a tumor antigen and that epitope $CEA_{653–667}$ could be used for immunotherapy against tumors expressing CEA.**

INTRODUCTION

CEA[3] is an M_r 180,000 glycoprotein that is an ideal TAA for the development of immunotherapy because it is produced by most colorectal, gastric, and pancreatic carcinomas (1). Furthermore, CEA is abundantly expressed by 50% of breast cancer and 70% of non-small cell lung carcinomas (2). Several proteins from the CEA gene family such as NCA and BGP are expressed in the normal cells (*e.g.*, neutrophils and granulocytes) and share regions of identical sequence with CEA (2). Moreover, CEA is also present (although at usually much lower concentrations) in the normal colon epithelium and in some fetal tissues. Therefore, CEA does not constitute tumor-specific antigen, creating concerns that it might either induce pathogenic immune responses to normal cells (*i.e.*, autoimmunity) or, alternatively, be nonimmunogenic because of immune tolerance.

The design and implementation of T-cell-based immunotherapy for cancer relies heavily in the identification of immunogenic T-cell peptide epitopes from TAAs capable of eliciting antitumor responses. Because MHC class I-restricted CTLs have a direct lytic effect on tumor cells, most efforts have focused on the identification of peptide epitopes capable of stimulating these types of responses. As a consequence, numerous CTL epitopes from CEA have been described, which were found to be restricted by commonly found MHC class I alleles such as HLA-A2, -A3, -A24, -B7, and -B27 (3–10). Therefore, the identification of CTL epitopes for CEA has opened the door to the development of epitope-based immunotherapy for CEA-expressing tumors.

Because HTLs play an important role both in the induction and maintenance of CTL responses, vaccines that activate both CTLs and HTLs should be more effective than vaccines that only target CTL responses (11). Experiments in animal model systems have demonstrated the importance of antigen-specific HTLs in the elimination of tumors by CTLs (12–16). Moreover, using an *in vitro* model of human antitumor rejection mediated by CTLs, we reported recently that the presence of HTLs was critical for the eradication of the tumor cells (17). These experiments demonstrated that only in the presence of HTLs were the CTLs able to expand during the effector phase of the immune

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[3] The abbreviations used are: CEA, carcinoembryonic antigen; TAA, tumor-associated antigen; NCA, non-cross-reactive antigen; BGP, biliary glycoprotein; HTL, helper T lymphocyte; EBV-LCL, EBV-transformed lymphoblastoid cell line; PBMC, peripheral blood mononuclear cell; IL, interleukin; Hpcal 1, hippocalcin-like 1 protein; DC, dendritic cell; APC, antigen-presenting cell.

response, resulting in an increase in the overall antitumor cytolytic activity. In view of this, one obvious way to improve vaccines designed to induce antitumor CTLs is to include in these vaccines MHC class II-restricted epitopes that would trigger HTL responses to TAAs. In agreement with this assessment, several groups including ours have devoted considerable efforts to defining T-helper epitopes for various TAAs such as tyrosinase, gp100, MART1/Melan-A, NY-ESO-1, p53, and HER2/*neu* (18–29). In some of these cases, the identified HTL epitopes were found to be highly promiscuous with regard to their MHC class II restriction, which would allow their use in large patient populations.

In the present study, we used a computer-based algorithm (30) to identify peptide sequences from CEA with potential promiscuous MHC class II binding characteristics. Using this approach, we selected peptide $CEA_{653-667}$, which was found to be effective in generating *in vitro* HTL responses restricted by the HLA-DR4, -DR7, and -DR9 alleles. More importantly, some of the peptide-generated HTLs were also capable of recognizing naturally processed antigen in the form of recombinant CEA protein or cell lysates prepared from CEA-positive tumors. The present findings should hold some value for the development of epitope-based vaccines to elicit CTL and HTL responses against CEA-expressing tumors.

MATERIALS AND METHODS

Cell Lines. EBV-LCLs were produced from PBMCs of HLA-typed volunteers using culture supernatant from the EBV-producing B95-8 cell line. Mouse fibroblast cell lines (L-cells) transfected and expressing individual human MHC class II molecules were kindly provided by Dr. R. W. Karr (Pfizer Global R&D, New London, CT) and by Dr. T. Sasazuki (Fukuoka, Japan). The colon adenocarcinoma cell line SW403, the Jurkat T-cell lymphoma, and the IL-2-dependent CTLL cell line were purchased from American Type Culture Collection (Manassas, VA). The colon carcinoma cell line WiDr was supplied by the Japanese Cancer Research Resources Bank (Tokyo, Japan).

Synthetic Peptide and Recombinant Antigens. Potential HLA-DR promiscuous $CD4^+$ T-cell epitopes were selected from the amino acid sequence of CEA using the algorithm tables for three HLA-DR alleles (*DRB1*0101*, *DRB1*0401*, and *DRB1*0701*) described by Southwood *et al.* (30). The selected peptide, $CEA_{653-667}$ (YACFVSNLATGRNNS), which displayed high algorithm scores, was synthesized by solid phase organic chemistry and purified by high-pressure liquid chromatography. The purity (>80%) and identity of peptide were assessed by high-pressure liquid chromatography. Recombinant CEA protein was purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant 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.

***In Vitro* Induction of Antigen-specific HTL Lines with Synthetic Peptide.** The procedure selected for the generation of tumor antigen-reactive HTL lines using peptide-stimulated PBMCs has been described in detail (21–23). Briefly, DCs were produced in tissue culture from adherent monocytes that were cultured for 7 days at 37°C in a humidified CO_2 (5%) incubator in the presence of 50 ng/ml granulocyte/macrophage-colony stimulating factor and 1000 IU/ml IL-4. Peptide-pulsed DCs (3 μg/ml for 2 h at room temperature) were irradiated (4200 rad) and cocultured with autologous purified $CD4^+$ T cells (using antibody-coated magnetic microbeads from Miltenyi Biotech, Auburn, CA) in round-bottomed, 96-well culture plates. One week later, the $CD4^+$ T cells were restimulated with peptide-pulsed irradiated autologous PBMCs, and 2 days later, human recombinant IL-2 was added at a final concentration of 10 IU/ml. One week later, the T-cells were tested for their proliferative responses to peptide as described below. Those cultures exhibiting a proliferative response to peptide (at least 3-fold over background) were expanded in 24- or 48-well plates by weekly restimulation with peptides and irradiated autologous PBMCs. Complete culture medium for all procedures consisted of RPMI 1640 supplemented with 5% human male AB serum, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 μg/ml gentamicin. The Institutional Review Board on Human Subjects (Mayo Foundation) approved this research, and informed consent for blood donation was obtained from all volunteers.

Measurement of Antigen-specific Responses with HTL Lines. $CD4^+$ T cells (3×10^4/well) were mixed with irradiated APCs in the presence of various concentrations of antigen (peptides, recombinant proteins, or tumor lysates) in 96-well culture plates. APCs consisted of either PBMCs (1×10^5/well), HLA-DR-expressing L-cells (3×10^4/well), EBV-LCLs (3×10^4/well), or DCs (5×10^3/well). Tumor cell lysates were prepared by three freeze-thaw cycles of 1×10^8 tumor cells, resuspended in 1 ml of serum-free RPMI 1640. Lysates were used as a source of antigen at 5×10^5 cell equivalents/ml. Culture supernatants were collected after 48 h for measuring antigen-induced lymphokine (granulocyte/macrophage-colony stimulating factor) production by the HTLs using ELISA kits (PharMingen, San Diego, CA). Cell proliferation assays were incubated at 37°C for 72 h, and during the final 16 h, each well was pulsed with 0.5 μCi/well of [^{3}H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ). The radioactivity incorporated into DNA, which correlates with cell proliferation, was measured in a liquid scintillation counter after harvesting the cell cultures onto glass fiber filters. To determine MHC restriction molecules involved in antigen presentation, blocking of the antigen-induced proliferative 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; Ref. 31) or anti-HLA-A, -B, -C monoclonal antibody W6/32(IgG2a, American Type Culture Collection). To evaluate the effect of antigen-specific antibodies on the response of HTLs to CEA, we added anti-CEA monoclonal antibody MD06 (IgG1; Takara, Otsu, Japan). All antibodies were used at a final concentration of 10 μg/ml throughout the 48-h incubation period. All assessments of proliferative responses or ELISA were carried out at least in triplicate, and results correspond to the mean values with the SD of the mean.

RESULTS

Selection of Potential HTL Epitopes for CEA. Because our goal was to identify promiscuous MHC class II HTL epitopes for CEA, we first examined the amino acid sequence of this TAA for the presence of peptides containing binding motifs for *HLA-DRB1*0101, DRB1*0401,* and *DRB1*0701* (referred to as HLA-DR1, -DR4, and DR7 in the remaining text of this work), which are commonly found in the general population. The binding motifs that we used are based on the algorithm values described by Southwood *et al.* (30), which calculate the potential (predicted) binding interactions of primary and secondary anchors of a 9-amino acid "core region" for each of the three MHC class II alleles. This approach has been very successful in our hands, allowing us to identify several promiscuous HTL epitopes from TAAs such as HER2/*neu*, gp100, and MAGE-A3 (21–23). The four highest ranking, potentially promiscuous core sequences that were identified using this method were $CEA_{97–110}$, $CEA_{116–130}$, $CEA_{653–667}$, and $CEA_{665–679}$. The amino acid sequences of $CEA_{97–110}$ and $CEA_{116–130}$ are almost identical (only one amino acid difference) to the sequence found on the two closely related proteins of CEA, BGP, and NCA, which are widely expressed throughout the organism. Thus, we decided not to select these epitopes for further analysis because of the possibility of the existence of immune tolerance or, alternatively, concerns of inducing generalized autoimmune toxicity. The potential HTL epitope $CEA_{665–679}$ is destroyed during posttranslational modification of the CEA protein by protease cleavage at position 676 (32, 33), creating the uncertainty of whether this peptide will exist in sufficient amounts in APCs to function as an HTL epitope. On the other hand, the sequence represented by peptide $CEA_{653–667}$ was found to be present in CEA and not in BGP or NCA, making it an attractive candidate as a promiscuous epitope for tumor-reactive HTLs. We thus decided to focus our efforts in peptide $CEA_{653–667}$ to evaluate whether it could be capable of inducing antigen-specific HTL responses after *in vitro* T-cell immunization.

T-Cell Responses to a Peptide Epitope from CEA. Peptide $CEA_{653–667}$ was synthesized and tested for its ability to stimulate T-cell responses using PBMCs isolated from four healthy, MHC class II-typed individuals (HLA-DR1/13, HLA-DR4/15, HLA-DR4/9, and HLA-DR7/17). Purified $CD4^+$ T cells were stimulated in primary cultures using peptide-pulsed autologous DCs as APCs, as described in "Materials and Methods." After three to four cycles of peptide restimulation using autologous irradiated PBMCs as APCs, the lymphocyte cultures were then tested for their capacity to respond to the peptide presented by autologous PBMCs as APCs. Those cultures that exhibited at least a 3-fold increase of proliferative response to peptide (data not shown) were selected and expanded for further analyses. For 3 of the 4 individuals, HTL lines were obtained and analyzed for their MHC restriction patterns. In the case of the DR4/DR9 individual, significant T-cell responses to peptide $CEA_{653–667}$ were observed when autologous PBMCs were used as APCs, which could be inhibited to a great extent by anti-HLA-DR antibodies but not by anti-MHC class I antibodies (Fig. 1*a*). These T cells did not respond significantly to an irrelevant peptide (data not shown). When mouse fibroblasts (L-cells) transfected with HLA-DR4, -DR9, or -DR53 were used as APCs, the HTLs from this individual recognized peptide $CEA_{653–667}$ only when presented by the L-DR9 cells (Fig. 1*b*). To evaluate the overall affinity (avidity) of the HLA-DR9-restricted HTLs for its ligand, peptide titration curves were performed using various types of autologous APCs (DCs, PBMCs, and EBV-LCLs) and L-DR9 cells. These results show that the HTL line displayed high avidity for peptide $CEA_{653–667}$ because <1 μg/ml of peptide was required to attain 50% of maximal response when autologous DCs, PBMCs, or EBV-LCLs were used as APCs (Fig. 1*c*). These results also indicate that although DCs were the most potent type of APCs, requiring ~10 ng/ml of peptide to produce 50% of maximal response, the L-DR9 cells were ~200-fold less potent as APCs, possibly because of the lack of human adhesion molecules.

From the DR4/15 and DR7/17 individuals, HTL cultures were obtained, which proliferated to peptide $CEA_{653–667}$ presented by L-DR4 and L-DR7 APCs, respectively (Fig. 2), indicating that this epitope has some degree of promiscuity. However, these HTLs did not expand sufficiently to allow us to conduct additional experiments. Lastly, peptide $CEA_{653–667}$ was unable to trigger T-cell responses in the HLA-DR1/DR13 individual (data not shown).

Recognition of Processed Tumor Antigens by $CEA_{653–667}$-reactive HTLs. The results presented above indicate that the peptide $CEA_{653–667}$ was indeed capable of inducing $CD4^+$ T-cell responses. However, it is important to determine whether the peptide-reactive T cells are capable of recognizing the naturally processed antigen, in this case, the CEA protein. Thus, APCs (DCs or monocytes) that capture the CEA protein (or cell lysates from CEA+ tumors) would be expected to process the antigen to produce a peptide corresponding to $CEA_{653–667}$ (or a closely related species), which would bind to MHC class II molecules to be presented to the T cells. Only under these circumstances can one be certain that the predicted T-cell epitope will be biologically relevant for developing immunotherapy against CEA-expressing tumors. Consequently, we first proceeded to test the capacity of the HLA-DR9-restricted, $CEA_{653–667}$-specific T-cell line to recognize naturally processed antigen in the form of recombinant CEA protein. As shown in Fig. 3*a*, the $CEA_{653–667}$-reactive T-cell line responded well to recombinant CEA protein but not to a control antigen, recombinant Hpcal 1, when autologous DCs were used as APCs. These results demonstrate that the epitope represented by peptide $CEA_{653–667}$ is produced by the antigen-processing pathway occurring on APCs. Because it would be more significant to assess whether the APCs will capture antigen derived from dead tumor cells expressing CEA and appropriately present the epitope to the HTLs, we examined this possibility. The $CEA_{653–667}$-reactive HTLs were evaluated for their capacity to recognize naturally processed tumor antigen using DCs that were fed with dead CEA-expressing tumor cells (in this case, freeze/thaw cell lysates). The data presented in Fig. 3*b* demonstrate that the $CEA_{653–667}$-reactive T-cell line was able to respond in a dose-dependent manner to DCs presenting cell lysates from two CEA-positive tumors (SW403 and WiDr) but not with DCs presenting a lysate from a CEA-negative T-cell lymphoma (Jurkat).

The capacity of the HTLs to recognize naturally processed

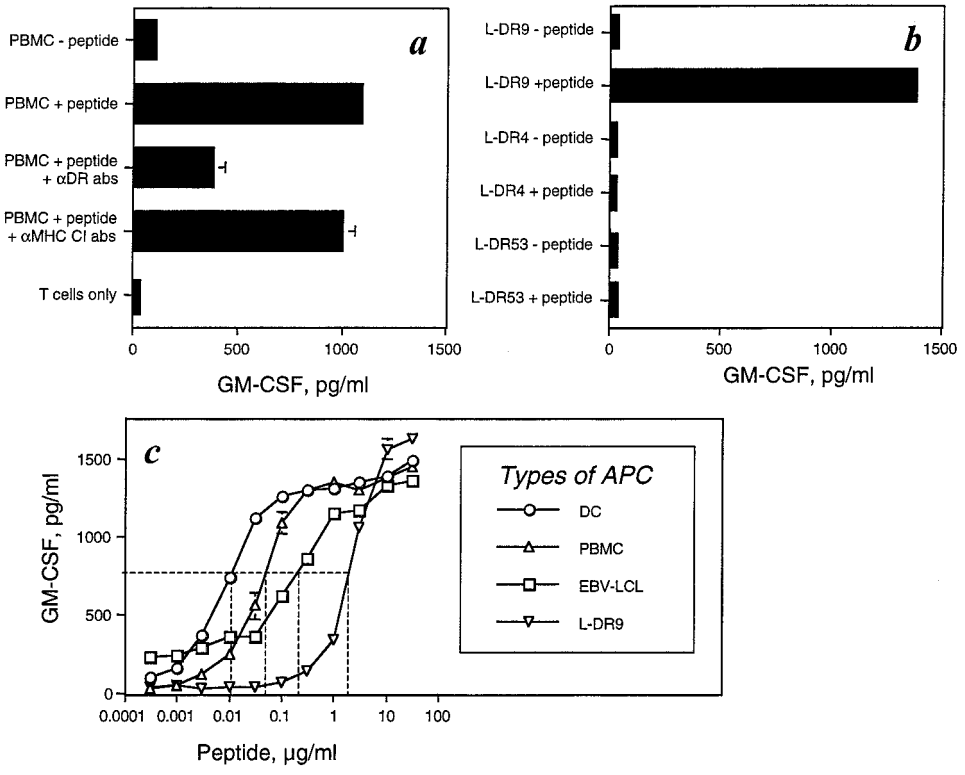

Fig. 1 HLA-DR9-restricted T-cell responses to peptide $CEA_{653–667}$. An HTL line was selected from an HLA-DR4/DR9 normal individual by weekly stimulation of CD4+ T cells with peptide and APCs as described in "Materials and Methods." Using autologous PBMCs as APCs (*a*), the T-cell response to peptide $CEA_{653–667}$ was inhibited by anti-HLA-DR monoclonal antibodies (α*DR abs*, L243 at 10 μg/ml) but not by anti-MHC class I antibodies (α*MHC CI abs*, W6/32 at 10 μg/ml). When mouse fibroblasts transfected with human HLA-DR genes were used as APC (*b*), it became evident that the HTL line recognized peptide $CEA_{653–667}$ (used at 3 μg/ml) in the context of HLA-DR9. Peptide dose-curve responses were performed to estimate the overall avidity of the HTLs for peptide $CEA_{653–667}$ using various types of APCs (*c*). *Dashed lines*, the peptide concentration required to obtain 50% of the maximal response (~0.01 μg/ml with DCs, ~0.05 μg/ml with PBMCs, ~0.2 μg/ml with EBV-LCLs, and ~2.0 μg/ml with L-DR9 cells). Values shown are the means of triplicate determinations; *bars*, STD. Columns and symbols without bars have SDs too small to appear in the figure.

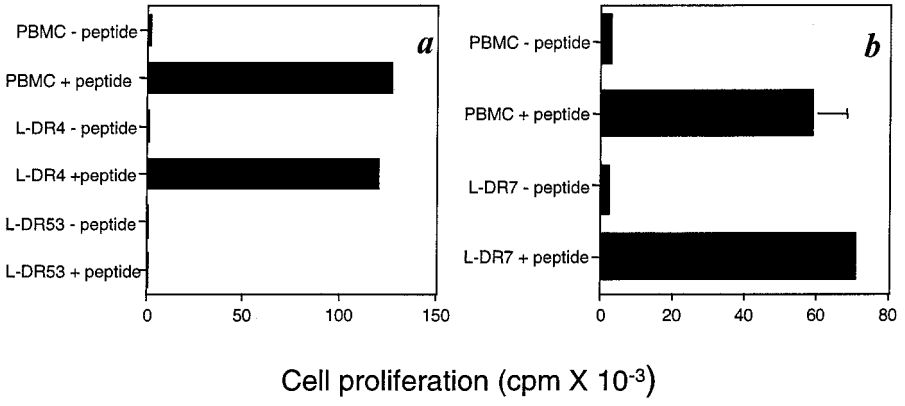

Fig. 2 HLA-DR4 and -DR7-resricted T-cell responses to peptide $CEA_{653–667}$. HTL lines were selected from DR4/15 (*a*) and DR7/17 (*b*) normal donors by weekly stimulation of CD4+ T cells with peptide and APCs. Results represent the peptide-induced proliferative responses to 3 μg/ml of $CEA_{653–667}$ in the presence of various types of irradiated APCs. Values shown are the means of triplicate sample; *bars*, STD. Columns without error bars have SDs too small to appear in the figure.

CEA in the form of tumor lysates presented by DCs was inhibited by anti-HLA-DR antibodies (but not by anti-MHC class I antibodies), confirming that the processed T-cell epitope represented by $CEA_{653–667}$ was presented in the context of MHC class II molecules (Fig. 4). Furthermore, the T-cell responses to the tumor lysates were substantially enhanced by the addition of anti-CEA antibodies (Fig. 4), which presumably increases the delivery of antigen to the APCs via the formation of immune complexes that are captured through surface Fc receptors (34, 35). The enhancement by the anti-CEA antibodies was antigen specific because no effects were observed when lysates from CEA-negative tumors (Jurkat) were used.

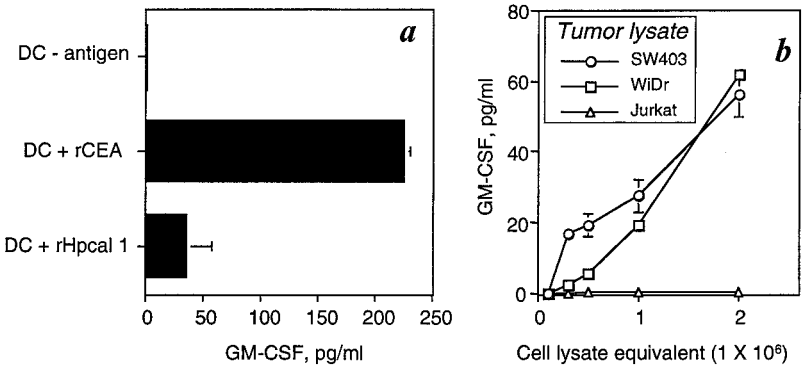

Fig. 3 Recognition of naturally processed antigen by the HLA-DR9-restricted $CEA_{653-667}$-specific HTL line. Using autologous DCs as APCs, the peptide-induced HTLs were able to recognize recombinant CEA protein (*a*) or various amounts of cell lysate from tumors expressing CEA (*b*). Values shown are the means of triplicate determinations; *bars*, SD. Columns and symbols without error bars have SDs too small to appear in the figure.

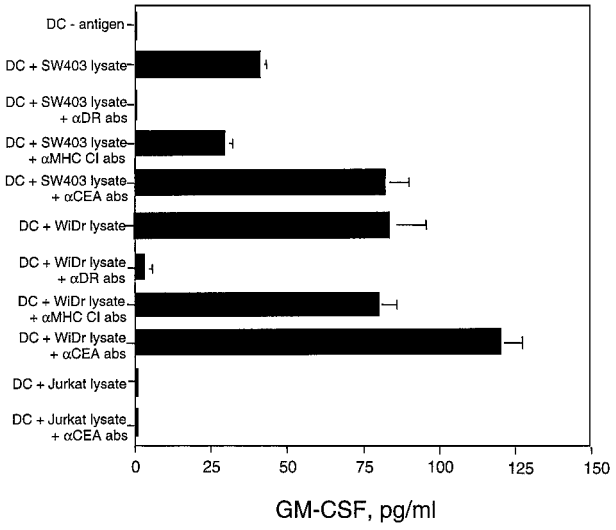

Fig. 4 Antibody-mediated blocking or enhancement in the responses of HTL to naturally processed antigen. Using autologous DCs as APCs, the effect of antibodies specific for HLA-DR (α*DR abs*), MHC class I (α*MHC CI abs*), or CEA (α*CEA abs*) were evaluated in the response of the HLA-DR9-restricted HTL to lysates from SW403 (*CEA*+), WiDr (*CEA*+), or Jurkat (*CEA*−). Values shown are the means of triplicate determinations; *bars*, SD. Columns without error bars have SDs too small to appear in the figure.

DISCUSSION

Although numerous CEA epitopes for MHC class I-restricted CTLs have been identified (3–10), to our knowledge this is the first report of a peptide epitope capable of stimulating MHC class II-restricted HTL responses. This epitope was discovered using a computer-based algorithm (30) that has also enabled us to identify HTL epitopes from other TAAs such as HER2/*neu*, gp100, and MAGE3 (21–23). Although the epitope represented by peptide $CEA_{653-667}$ was selected on the basis of potentially being a peptide capable of binding to HLA-DR1, -DR4, and -DR7, the T-cell responses induced by this peptide in our present studies were found to be restricted by HLA-DR4, -DR7, and -DR9. From the limited number of experiments done here, we cannot exclude the possibility that peptide $CEA_{653-667}$ will not induce HLA-DR1-restricted T-cell responses. The observation that HLA-DR9-restricted responses were produced by peptide $CEA_{653-667}$ is not unexpected because we and others have observed that peptides predicted to be promiscuous epitopes using the "Southwood algorithm" in many cases extend their restriction capacity beyond the three alleles (HLA-DR1, -DR4, and -DR7) that are considered by this algorithm (21, 22, 30, 36). Epitopes identified by this method have been found to be presented by numerous alleles such as HLA-DR1, -DR4, -DR7, -DR9, -DR11, -DR13, -DR15, -DR16, -DR51, -DR52, -DR53, -DQ2, -DQ6, or -DQ7. Moreover, peptide $CEA_{653-667}$ also scored high using a predictive algorithm for promiscuous MHC class II binders that we have developed (data not shown; Ref. 37). Thus, it is probable that peptide $CEA_{653-667}$ could be recognized by HTLs in the context of additional alleles besides HLA-DR4, -DR7, and -DR9, but experimental data will be required to test such possibility.

Recognition of naturally processed antigen is a hallmark that the predicted epitope will be relevant for vaccine/immunotherapy development. In many cases, peptide-reactive HTLs are not capable of recognizing APCs that ingest and process the antigen into MHC class II-binding peptides. Possibilities for the lack of recognition of naturally processed antigen by peptide-reactive T cells could be: (*a*) presence of partially deprotected peptide impurities, which could be very immunogenic; (*b*) the destruction of the corresponding peptide if it is cleaved inappropriately by proteases during processing (*i.e.*, "cryptic epitopes"); and (*c*) insufficient density of specific MHC peptide complexes on the surface of the APCs to allow T-cell activation. The last possibility could be the result of low amounts of CEA protein captured by the APCs or if the peptide does not bind with sufficient affinity to the MHC molecule to generate stable peptide/MHC complexes. The data presented herein demonstrate that, at least in the case of T-cell responses to peptide $CEA_{653-667}$ that are restricted by HLA-DR9, the HTLs were quite effective in recognizing naturally processed CEA in the form of recombinant protein or cell lysates from CEA+ tumors. Although it seems reasonable that the HLA-DR4- and -DR7-restricted HTLs would also recognize the naturally processed CEA epitope, this possibility could not be assessed because the T-cell lines were not sufficiently stable to allow us to carry out these determinations.

Interestingly, the newly identified HTL CEA epitope was found to overlap with the previously described HLA-A24-restricted CTL epitope, $CEA_{652-660}$ (7), which would allow the

use of a relatively small peptide ($CEA_{652-667}$) as a vaccine designed to elicit both CTL and HTL responses in some individuals. Such a vaccine would be quite useful in the Japanese population, where HLA-A24, -DR4, and -DR9 are common MHC alleles.

In addition to potentially being a promiscuous HTL epitope, we deliberately selected the $CEA_{653-667}$ sequence because it was absent in BGP and NCA. This decision served two purposes: (*a*) we were concerned that peptides corresponding to CEA sequences that are identical (or similar) to BGP or NCA would not be immunogenic because immune tolerance is likely to exist to widely expressed proteins such as BGP and NCA; and (*b*) most importantly, we wanted to diminish the chances of inducing serious autoimmune pathology if a vaccine containing a cross-reactive T-cell epitope would be effective in overcoming immune tolerance because BGP and NCA are widely expressed in various tissues. Nevertheless, we are conscious that a vaccine based on peptide $CEA_{653-667}$ could still induce autoimmune pathology because CEA is expressed in small amounts in the gut. However, because no reports of serious adverse events have occurred in numerous vaccine studies using CEA as a TAA (38–46), we are strongly hopeful that a therapeutic vaccine for tumors expressing CEA, which incorporates the HTL epitope identified here, would increase the chances of inducing effective and long-lasting immune responses that may lead to clinical benefit.

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