ABSTRACT

Tumor cells can be effectively recognized and eliminated by CTLs. One approach for the development of CTL-based cancer immunotherapy for solid tumors requires the use of the appropriate immunogenic peptide epitopes that are derived from defined tumor-associated antigens. Because CTL peptide epitopes are restricted to specific MHC alleles, to design immunotherapies for the general population it is necessary to identify epitopes for the most commonly found human MHC alleles. The identification of such epitopes has been based on MHC-peptide-binding assays, which are readily available in the public domain (Internet), to identify HLA-B7-restricted CTL epitopes for carcinoembryonic antigen (CEA). These algorithms identified three candidate peptides that we studied for their capacity to induce CTL responses in vitro using lymphocytes from HLA-B7+ normal blood donors. The results show that one of these peptides, CEA9 632 (IPQQHTQVL) was efficient in the induction of primary CTL responses when dendritic cells were used as antigen-presenting cells. These CTLs were efficient in killing tumor cells that express HLA-B7 and produce CEA. The identification of this HLA-B7-restricted CTL epitope will be useful for the design of ethnically unbiased, widely applicable immunotherapies for common solid epithelial tumors expressing CEA. Moreover, our strategy of identifying MHC class I-restricted CTL epitopes without the need of peptide/HLA-binding assays provides a convenient and cost-saving alternative approach to previous methods.

INTRODUCTION

CTLs are recognized as the most direct and effective elements of the immune system that are capable of generating antitumor immune responses (1–5). Tumor cells expressing the appropriate CTL antigens can be effectively recognized and destroyed by these immune effector cells, which may result in dramatic clinical responses (6–9). Both the adoptive transfer of tumor-reactive CTLs and active immunization designed to elicit CTL responses have been reported to lead to significant therapeutic antitumor responses in patients with malignant melanoma (7–10). However, these promising approaches and their applicability to other tumor types besides melanoma are somewhat restricted because of the limited number of tumor antigens, or epitopes, for CTLs that are currently available.

The CTL epitopes on tumor cells are formed of MHC molecules that bind peptides derived from the intracellular processing of proteins (11–13), some of which function as TAAs. CEA is a M, 180,000 glycoprotein that is an ideal TAA because it is extensively expressed on the vast majority of colorectal, gastric, and pancreatic carcinomas (14). In addition, CEA is found in 50% of breast cancer and 70% of non-small cell lung carcinomas (15). CEA does not constitute a tumor-specific antigen because it is also present (although at usually much lower concentrations) in the normal colon epithelium and in some fetal tissues. Furthermore, circulating CEA can be detected in the great majority of patients with CEA-positive tumors and has been used to monitor responses to therapy and disease progression. The ability of CTLs to recognize epitopes derived from CEA has been demonstrated both in cancer patients (16) and in normal individuals whose cells have been immunized in vitro with MHC-binding peptides from CEA (17–19). Using peptide-pulsed DCs as antigen-presenting cells, we have been able to generate in vitro CTLs that recognize tumor cells that express CEA. As the result of these experiments, CEA CTL epitopes restricted by HLA-A2, -A3, and -A24 have been successfully identified (17–19). These frequently found alleles cover approximately 60–70% of the general population. To further extend the potential population coverage for a CTL-based approach for immunotherapy of CEA-expressing tumors, we have proceeded to identify additional CTL epitopes for this TAA. Here we report that peptide CEA9 632 (IPQQHTQVL), which was identified by the combination of two predictive algorithms, was successful in generating in vitro CTL responses restricted by HLA-B7 (B*0702), an allele that is found in approximately 10–16% of humans. Most importantly, these CTLs were capable of recognizing and killing CEA+, HLA-B7+ tumor cells, which indicated that the epitope represented by this immunogenic peptide is being processed and presented in the context of HLA-B7. To our knowledge, this is the first example of the successful identification of CTL epitope for a TAA that is restricted by HLA-B7 using predictive algorithms. This approach could be of value for the selection of CTL epitopes to design ethnically unbiased therapeutic vaccines.

MATERIALS AND METHODS

Epitope Selection and Peptide Synthesis. We used the combination of two computer algorithms that exist in the public domain and are easily accessible through the Internet. The predictive algorithm, “BIMAS” developed by K. C. Parker and collaborators (20), is available at a web site of the NIH. This computer algorithm ranks potential MHC binders according to the predictive half-time dissociation of peptide/MHC complexes. The second algorithm, “SYFPEITHI,” was developed by H. G. Rammensee et al. (21) and ranks the peptides according to a score that takes into account the presence of primary and secondary MHC-binding anchor residues. The amino acid sequence of CEA was analyzed on both of the computer programs for the existence of 9-amino acid peptides predicted to bind to HLA-B*0702. The number of candidate peptides was narrowed down according to these criteria: (a) those peptides that did not contain canonical HLA-B7-binding anchors, Pro at position 2 and Leu or Ile at position 9 (21), were eliminated from the list of potential CTL epitopes; (b) the algorithm scores of the remaining peptides were compared with those obtained using four known 9-residue HLA-B7-restricted CTL epitopes (22–25), and algorithm cutoff values were established. The analysis resulted in three candidate peptides for HLA-B7-restricted CTL epitopes: CEA9 632 (LPVSRLQQL), CEA9 541 (NPPAQYSLW), and CEA9 442 (IPQQHTQVL). These peptides were synthesized according to standard solid-phase synthetic methods using Applied Biosystems apparatus and were purified by high-performance liquid chromatography. The purity...
The EBV-transformed B-cell line JY (homozygous for HLA-A2 and -B7) was used as target for CTL-mediated cytolysis to demonstrate peptide reactivity. The JY cells were kept in tissue culture using RPMI 1640 supplemented with 10% fetal bovine serum (v/v), L-glutamine, nonessential amino acids, sodium pyruvate, and gentamicin (complete RPMI medium). The colon adenocarcinoma cell line SW403 (HLA-B7+), the breast cell line HBL-100 (HLA-B7+), and the natural killer-sensitive K562 erythroleukemia line were all obtained from American Type Culture Collection (Manassas, VA) and were maintained in tissue culture as recommended by the supplier. The melanoma cell line ME180 was provided by Dr. Y. Kawakami (National Cancer Institute, NIH, Bethesda, MD) and was grown in complete RPMI medium. All of the culture materials were purchased from Life Technologies Inc. (Rockville, MD). To increase the level of MHC class I expression, tumor cell lines (except for JY) were treated with 100 units/ml IFN-γ for 48 h before the CTL cytototoxicity assays.

**In Vitro Generation of Tumor-reactive CTLs.** DCs were generated from CD14+ precursor cells as described (26–28) and were used as APC to immunize CTL precursors with the candidate synthetic peptides. Briefly, purified monocytes were cultured for 7 days in the presence of 50ng/ml GM-CSF and 1000 units/ml rIL-4 in complete RPMI medium. The tissue culture-generated DCs were pulsed with 40 μg/ml of synthetic peptides together with 3 μg/ml β2-microglobulin in PBS containing 1% BSA for 4 h at room temperature. The peptide-pulsed DCs were washed twice and irradiated (4200 rads). The peptide-pulsed DCs were then mixed with autologous purified CD8+ T cells (puriﬁed with Miltenyi immuno-magnetic beads by positive selection) at 1:20 (DC:T-cell) ratio. The CTL immunization cultures were done in 48-well plates, for which each well contained 0.25 × 10^6 DC cells and 5 × 10^5 CD8+ T-cells in 0.5 ml of complete RPMI medium containing 5% human AB serum instead of fetal bovine serum. This medium was supplemented with 10 ng/ml rIL-7. One day later, 10 ng/ml rIL-10 were added to the cultures to increase the efficiency of CTL induction. On days 7 and 14, the T-cell cultures were individually restimulated with peptide-pulsed irradiated autologous APC (adherent monocytes) as described, adding IL-10 on the following day. Starting on day 9, the T-cell cultures were fed with fresh medium containing 10 units/ml IL-2 every 2–3 days. The first screening cytotoxicity assay was performed after three rounds of peptide stimulation. And those cultures that exhibited cytotoxic activity toward peptide-pulsed JY cells (>20% lysis as compared with the unpulsed target cells) were selected and expanded in tissue culture for further analysis. CTL clones were established by limiting dilution using monoclonal anti-CD3 antibody and feeder cells as described previously (26) The Institutional Review Board (IRB) on Human Subjects (Mayo Foundation) approved this research, and informed consent for blood donation was obtained from all of the volunteers.

**CTL Cytotoxicity Assays.** Cytotoxic activity of CTLs was determined in a standard 4–6 h ^51Cr release assay as described (29). Peptide-pulsed targets were prepared by incubating JY cells with 10 μg/ml peptides at 37°C overnight. Adherent tumor cells were removed from culture flask with trypsin-EDTA immediately before ^51Cr-labeling. Target cells were labeled with 300 μCi[^51Cr]sodium chromate (Amersham Pharmacia Biotech, Piscataway, NJ) for 1–2 h at 37°C in a water bath. Various numbers of effector cells were mixed with 2 × 10^4 labeled targets at different E:T ratios in 96-round-bottomed-well plates at a final volume of 0.2 ml. After 4–6 h incubation at 37°C, 30 μl of supernatant were collected from each well and the percentage of specific lysis was determined according to the formula:

\[
\frac{cpm\ of\ the\ test\ sample - cpm\ of\ spontaneous\ release}{cpm\ of\ the\ maximal\ release - cpm\ of\ spontaneous\ release} \times 100
\]

For the first screening assay, the labeled target cells were mixed with cold (unlabeled) K562 cells at a 1:20, labeled-to-cold target ratio to decrease the nonspecific killing attributable to natural killer cells. Results show average specific lysis ± SE of triplicate determinations.

**Cold-Target Inhibition and Antibody Blocking Assays.** Antigen specificity was confirmed by cold-target inhibition assays by unlabeled peptide-pulsed JY cells to compete for the lysis of the labeled tumor cells expressing CEA. Percentage of inhibition lysis was calculated by the following formula:

\[
\frac{\%\ specific\ lysis\ without\ inhibitors - \%\ specific\ lysis\ with\ inhibitors}{\%\ specific\ lysis\ without\ inhibitors} \times 100
\]

For these experiments, JY cells were pulsed with 10 μg/ml peptide for 16 h at 37°C.

MHC restriction was determined by testing the capacity of anti-MHC class I (W6/32) and anti-MHC class II (9.3F10) monoclonal antibodies to inhibit the lysis of tumor lines. Target cells were preincubated in 10 μg/ml W6/32 or 10 μg/ml 9.3F10 for 1 h at 37°C before cytotoxicity assay. Antibodies were prepared from culture supernatants of hybridoma cells obtained from the American Type Culture Collection.

**RESULTS**

**Prediction of HLA-B7-binding Peptides from CEA.** To identify HLA-B7 restricted CTL epitopes for CEA, the amino acid sequence of CEA was analyzed using two computer algorithms (20, 21) that are available through the Internet. Both of these algorithms take into account the roles primary and secondary MHC-binding anchors and are based on a large database of known MHC-binding ligands and CTL epitopes. We focused on peptides of 9 amino acids because it has been reported that HLA-B*0702 (and other members of the HLA-B7 superfamily) favor binding peptides of this size as compared with peptides of 8 or 10 residues (30). However, there are some examples of peptides of 10 and 11 residues that serve as HLA-B7-restricted CTL epitopes. (31, 32). For our studies, the list of potential 9-mer epitopes was narrowed down using two criteria: (a) only peptides containing canonical HLA-B7-binding anchors (Pro in position 2 and Leu or Ile in position 9) were included; and (b) cutoff values for the algorithm scores were established by the analysis of four known HLA-B7 CTL epitopes (22–25). For the BIMAS algorithm (20), the cutoff score was 80; and for the SYFPEITHI algorithm (21), the set cutoff value was 21, both of which were the lowest scores found among the known HLA-B*0702 CTL epitopes. The final analysis resulted in three candidate peptides as potential HLA-B7-restricted CTL epitopes for CEA (shown in bold in Table 1). Table 1 presents the sequences and algorithm scores of the CEA peptides together with the four known CTL epitopes, which are specific for other antigens. Interestingly, the highest-ranking CEA peptide in both algorithms (LPVSFLRQL) was found repeated three times (at positions 185, 363, and 541) in the CEA sequence, which could potentially have an effect on the number of MHC-peptide complexes expressed on CEA+ tumor cells.

**Induction of CTLs Using CEA-derived Peptides and Identification of HLA-B7-restricted CTL Epitopes.** The three candidate peptides for CEA epitopes listed in Table 1 were synthesized and tested for their capacity to elicit in vitro primary CTL responses using cells from two HLA-B7+ normal volunteers. Purified CD8+ CTL precursors were first stimulated with autologous peptide-pulsed DCs in 48 individual cell cultures, which were set up for each volunteer, and, after two subsequent weekly antigen restimulations, the cultures were tested for their cytotoxic activity against peptide-pulsed JY target cells. Of the three peptides tested, only peptide CEA9_312 was able to induce antigen-specific CTL cultures in both HLA-B7+ normal individuals (Table 1). A total of three positive CTL cultures (two derived from one donor and one from the second donor) were obtained that killed the peptide-pulsed JY cells more than 20% above the level of lysis observed with the unpulsed JY cells (data of the initial screen not shown). Two of the CTL cultures (one from each HLA-B7 individual) were cloned by limiting dilution and were expanded for further analysis. The CEA-reactive CTL clones were capable of
and HBL-100, effectively process and present peptide CEA9632 in the context of the HLA-B7 allele. These results also indicate that the CEA-producing cell lines, SW403 (colon cancer, B71), HBL100 (breast, B71), SW403 (melanoma, B71), and 624mel (melanoma, B71) were tested for their ability to recognize tumor cells expressing CEA. Cytotoxicity was measured in a 4-h 51Cr release assay at various E:T ratios against the following target cells: □, JY pulsed with CEA9632; ■, JY without peptide; ●, SW403 (colon cancer, B71, CEA+); ○, HBL100 (breast, B71, CEA+); ▲, 624mel (melanoma, B71, CEA−). Results are expressed as mean ± SE of triplicate samples.

DISCUSSION

In the past, our laboratory has relied on the use of peptide-MHC-binding assays to select potential CTL epitopes from known TAAs, before these peptides are tested for in vitro CTL induction (17–19, 28, 33–36). Because the peptide-MHC-binding assays tend to be labor-intensive and somewhat costly (37), we have examined here the potential HLA-B7-restricted CTL epitopes for CEA.

Table 1 List of candidate HLA-B7 CTL epitopes for CEA

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Sequence</th>
<th>Position of first residue</th>
<th>BIMAS score</th>
<th>SYFPEITHI score</th>
<th>CTL activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predicted epitopes</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CEA</td>
<td>PPTTAKLTI</td>
<td>30</td>
<td>0.8</td>
<td>18</td>
<td>NT</td>
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<tr>
<td>CEA</td>
<td>GPAYSGREIL</td>
<td>92</td>
<td>8</td>
<td>18</td>
<td>NT</td>
</tr>
<tr>
<td>CEA</td>
<td>YPELPKPSI</td>
<td>141</td>
<td>3.6</td>
<td>20</td>
<td>NT</td>
</tr>
<tr>
<td>CEA</td>
<td>LPVSPRIQL</td>
<td>185/365/54</td>
<td>180</td>
<td>26</td>
<td>0/96</td>
</tr>
<tr>
<td>CEA</td>
<td>NPPAQYSWL</td>
<td>44</td>
<td>80</td>
<td>21</td>
<td>0/96</td>
</tr>
<tr>
<td>CEA</td>
<td>PPAQYSL</td>
<td>443</td>
<td>0.8</td>
<td>17</td>
<td>NT</td>
</tr>
<tr>
<td>CEA</td>
<td>IPQKHTQVL</td>
<td>632</td>
<td>80</td>
<td>23</td>
<td>3/96</td>
</tr>
<tr>
<td>Known epitopes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBNA-6</td>
<td>QPRAPIRPI</td>
<td>881</td>
<td>120</td>
<td>23</td>
<td>NT</td>
</tr>
<tr>
<td>EBNA-3</td>
<td>RPIPIRRL</td>
<td>247</td>
<td>80</td>
<td>21</td>
<td>NT</td>
</tr>
<tr>
<td>RU2-AS</td>
<td>LPRWPPQQ</td>
<td>ND</td>
<td>1200</td>
<td>26</td>
<td>NT</td>
</tr>
<tr>
<td>aCE (+10RF)</td>
<td>SRWPTCL</td>
<td>ND</td>
<td>800</td>
<td>23</td>
<td>NT</td>
</tr>
</tbody>
</table>

* In bold type: potential HLA-B7-restricted CTL epitopes for CEA.

** Algorithm score obtained using the computer program developed by Rammensee et al. (20).

† Algorithm score obtained using the computer program developed by Parker et al. (21).

‡ CTL activity is the number of positive CTL cultures in a total of 96 from two blood donors.

§ NT, not tested; ND, not determined.

* CTL epitope described in 22, 23.

† CTL epitope described in 24.

‡ CTL epitope described in 25.

Fig. 1. Peptide dose-response curve of CEA-specific CTLs. Radiolabeled JY cells were incubated with various concentrations of peptide CEA9632 for 30 min, and CTLs were added at a final E:T ratio of 10:1. Cytotoxicity was determined after 4 h as described in “Materials and Methods.” Results are expressed as mean ± SE of triplicate samples.

Fig. 2. CTLs induced with peptide CEA9632 can recognize tumors expressing CEA.

In the past, our laboratory has relied on the use of peptide-MHC-binding assays to select potential CTL epitopes from known TAAs, before these peptides are tested for in vitro CTL induction (17–19, 28, 33–36). Because the peptide-MHC-binding assays tend to be labor-intensive and somewhat costly (37), we have examined here the potential HLA-B7-restricted CTL epitopes for CEA.

Antigen Sequence Position of first residue BIMAS score SYFPEITHI score CTL activity

CEA PPTTAKLTI 30 0.8 18 NT
CEA GPAYSGREIL 92 8 18 NT
CEA YPELPKPSI 141 3.6 20 NT
CEA LPVSPRIQL 185/365/541 180 26 0/96
CEA NPPAQYSWL 442 80 21 0/96
CEA PPAQYSL 443 0.8 17 NT
CEA IPQKHTQVL 632 80 23 3/96

Known epitopes
EBNA-6 QPRAPIRPI 881 120 23 NT
EBNA-3 RPIPIRRL 247 80 21 NT
RU2-AS LPRWPPQQ 800 26 NT
aCE (+10RF) SRWPTCL ND 23 NT

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CEA-specific CTL (the clone shown in Fig. 2B) were significantly decreased by the W6/32 antibody but not by 9.3F10 (used here as a negative control), which indicates that the CTL epitope is presented by a MHC class I molecule on the tumor cells. This experiment was repeated one more time with similar results (not shown).

The antigen specificity of the CTLs that were induced with peptide CEA9632 was corroborated in a cold-target inhibition assay in which unlabeled (cold) JY cells that were pulsed or not with peptide CEA9632 were tested for their ability to block the lysis of radiolabeled CEA-expressing tumor cells. The data in Fig. 4 show that cytotoxicity against both SW403 (Fig. 4A) and HBL100 (Fig. 4B), was inhibited significantly by the CEA9632-pulsed JY cells but not by the unpulsed JY cells. These results demonstrate that CEA9632-specific CTL can recognize the naturally processed epitope expressed on the surface of CEA+ tumor cells.

HLA Restriction and Antigen-Specificity Analysis. To confirm that the recognition of the peptide CEA9632 by the CTL clones was HLA class I-restricted, we tested the blocking effects of anti-HLA class I (W6/32) and class II (9.3F10) monoclonal antibodies in the cytolytic activity of the T cells against CEA-expressing tumor cells. The killing of both SW403 (Fig. 3A) and HBL100 (Fig. 3B) by CEA9632-specific CTL (the clone shown in Fig. 2B) were significantly decreased by the W6/32 antibody but not by 9.3F10 (used here as a negative control), which indicates that the CTL epitope is presented by a MHC class I molecule on the tumor cells. This experiment was repeated one more time with similar results (not shown).

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Both antibodies were tested at 10 μg/ml peptide CEA9632 as an immunogen to induce antitumor CTLs from two HLA-B7 patients bearing CEA. The results demonstrate that peptide CEA9632 pulsed DCs stimulated tumor-reactive CTLs with peripheral blood mononuclear cells from two HLA-B7+ individuals. Furthermore, these CTLs killed CEA-expressing tumor cells in an antigen-specific, MHC-restricted fashion, which indicated that peptide CEA9632 is present as a complex with HLA-B7, on the surface of the tumor cells. Thus, based on these results, it would be reasonable to use peptide CEA9632 as an immunogen to induce antitumor CTL responses in HLA-B7+ patients bearing CEA+ tumors.

There are several possible reasons to explain the inability of peptides CEA9185/363/541 and CEA9442 to induce CTL responses in vitro: (a) although both of the computer-based algorithms predict that these peptides should bind to HLA-B7, the possibility does exist that they may not do so with sufficient affinity to form stable MHC-peptide complexes; (b) the amino acid sequences of both CEA9185/363/541 and CEA9442 are identical to sequences found on two closely related proteins of CEA, BGP and NCA. Because both BGP and NCA are expressed in a variety of normal tissues (15), it is possible that immune tolerance at the CTL level may exist if these peptides are good HLA-B7 binders. On the other hand, the sequence of CEA9632 (the immunogenic peptide) is not present on either BGP or NCA. This is significant not only because of the apparent lack of CTL tolerance of this epitope (at least in vitro) but also because it would be preferable not to induce CTL with anti-BGP or anti-NCA cross-reactivity in an immune-based therapy for CEA; and (c) from the present studies, we cannot discount the possibility that peptides CEA9185/363/541 and CEA9442 can be able to induce tumor-reactive CTL because we have tested them only twice (using peripheral blood mononuclear cells from two normal volunteers but in a total of 96 cell cultures). However, this possibility seems unlikely because the method for inducing in vitro CTL responses that is routinely used in our laboratory results in objective CTL responses in most of the cases in which peptides that represent CTL epitopes are used.

We believe that peptide CEA9632 could be valuable for the development of CTL-based immunotherapy against tumors that express CEA in cancer patients expressing the HLA-B*0702 allele. However, because there are several alleles for part of the HLA-B7 superfamily (which bind peptides with similar characteristics), it is possible that CEA9632 may also function as a CTL epitope with other closely related class I MHC alleles (e.g., B*3501, B*5101, B*5102, B*5301, and B*5401). This prospect seems likely because most of the residues of peptide CEA9632 (IPQHTQVLT) score as “favorable” or “neutral” using the B7-like supermotif described by Sidney et al. (30), with the exception of the Q2, which scores as “deleterious” for all of the alleles including B*0702. Nonetheless, it will be necessary to demonstrate that peptide CEA9632 can induce tumor-reactive CTLs with other members of the HLA-B7 superfamily before it is used for immunotherapy in patients expressing other alleles of the B7 superfamily besides B*0702. The possibility exists that CTL responses against peptide CEA9632 in cancer patients may be difficult to obtain if immune tolerance at the CTL level takes place because of the presence of large amounts of antigen produced by the patients’ tumors. In this case, it may be possible to overcome tolerance by using peptide analogues as described previously (38), or one may have to look for subdominant epitopes in which tolerance may not be as strong.

Numerous peptides corresponding to tumor-reactive CTL epitopes have already been used for immunotherapy in clinical studies in cancer patients with mixed results (7, 8, 39–42). Peptide vaccination (mostly in malignant melanoma), either with or without adjuvants, has been reported to generate objective tumor responses, which in some cases correlates with CTL activity. Furthermore, immunization with peptide-pulsed DCs appears to be an effective way of inducing CTLs and antitumor immunity both in human trials (7, 9) and with animal models.
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Use of Two Predictive Algorithms of the World Wide Web for the Identification of Tumor-reactive T-Cell Epitopes

Jun Lu and Esteban Celis


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