Generation of Cytotoxic T Lymphocytes against Native and Altered Peptides of Human Leukocyte Antigen-A*0201 Restricted Epitopes from the Human Epithelial Cell Adhesion Molecule

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ABSTRACT

A growing number of human tumor antigens have been described that can be recognized by CTLs in a MHC class I restricted fashion. The epithelial cell adhesion molecule (Ep-CAM) is expressed in a variety of human tumors and has attracted attention as a therapeutic target for monoclonal antibody serotherapy. We have identified immunogenic peptides derived from Ep-CAM, that bind to human leukocyte antigen-A*0201 and elicit strong peptide-specific human CTL responses, demonstrating that there is an effective T-cell repertoire against these Ep-CAM-derived peptides that can be recruited. Alterations to these peptides were made to increase their binding affinity to MHC class I molecules. The use of such “heteroclitic” peptides allowed generation of cytotoxic T cells that demonstrated increased killing of target cells pulsed not only with the heteroclitic but also with the native peptide. Most important, CTL cell lines that are generated against these peptides specifically lyse epithelial tumor cells expressing Ep-CAM but not normal hematopoietic or bronchial epithelial cells.

INTRODUCTION

There is growing evidence for both humoral and cellular immune recognition of cancer by the autologous human host (1, 2). Many previously defined tumor antigens have been identified by cloning the epitopes recognized by CTLs (3) and serological expression cloning of recombinant cDNA libraries of human tumors (2). CTLs are perhaps the most specialized and effective element of antitumor immune responses (4–6). CTLs recognize antigen in the form of small peptide epitopes bound to specific allelic products of the MHC (7, 8). A correlation has been demonstrated between immunogenicity and peptide binding affinity to class I MHC molecules for epitopes from viral antigens and for peptides from immunoglobulin (9–11). Introduction of conservative modifications at particular amino acid positions within the peptide increases HLA-A*0201 binding affinity (12, 13) and enhances recruitment of the T-cell repertoire against peptides with nondominant anchor residues (10). Improved induction of tumor-reactive CTLs with peptides modified at HLA-A*0201-binding residues has subsequently been demonstrated in human cancers (14).

Several categories of auto-immunogenic tumor antigens, including differentiation antigens (15), individual antigens caused by point mutations (16), and tumor-associated antigens such as MAGE (17) have been characterized. Some of these have already demonstrated favorable results in clinical trials (18, 19). However, the molecular characterization of additional CTL-defined tumor antigens is needed to develop polyvalent vaccines with sufficient immunotherapeutic effects, because immunoselection of antigen-negative tumor cell variants has been observed during peptide vaccination (20). Recently, novel bioinformatics approaches have identified a variety of immunogenic peptides derived from human tumor epitopes (21, 22) that elicit cytotoxic T-cell responses.

The Ep-CAM (11) also called EGP-2, 17–1A, GA733–2, or KSA (23–25), mediates Ca2+-independent homotypic cell-cell adhesion and is expressed on cells lining the lateral membrane of normal colon and other normal epithelial tissues (26). Ep-CAM is overexpressed in small cell lung cancer (27) and many adenocarcinomas of diverse histological origin and has attracted attention as a target for immunotherapy of lung, breast, and colon cancer (reviewed in Ref. 28). In patients with colorectal cancer expressing Ep-CAM, treatment with the mAb 17–1A was found in a randomized study to reduce mortality and recurrence by 32 and 23%, respectively (29, 30). An immunotoxin composed of the mAb MOC31 and a recombinant form of Pseudomonas exotoxin A demonstrated selective antitumor activity against Ep-CAM-expressing small cell lung cancer and lung adenocarcinoma cell lines in vitro and in xenograft models (31).

Recently, one Ep-CAM-derived nonamer peptide (263–271) was shown to induce peptide-specific cytotoxic T-cell responses in a HLA-A2 transgenic mouse model (32). Because the sequence of the Ep-CAM peptide examined differs from murine and human peptides at positions 3 and 6 (mouse GLTAGHAV, human GLKAGV1AV), the question arises whether this peptide is immunogenic only because no tolerance to this peptide motif developed in the mouse. Recently a T-cell response against this HLA-A2-restricted peptide was detected in patients with colorectal cancer (33). However, in this study, the effectiveness of these Ep-CAM-reactive T cells was not assessed. Here, we use a bioinformatics approach (34, 35) to predict for HLA-binding peptides from Ep-CAM and a cellular system (36) to analyze human T-cell responses against such potential antigenic peptides. Because CTL might be difficult to generate against low or intermediate affinity self-peptides (10, 37), we also tested highly conservative single amino acid substitution within the peptide sequence to increase HLA-A2-binding affinity. We demonstrate that human CTLs can be readily generated against two novel human Ep-CAM-derived peptides and that such CTLs are capable of killing tumor cells in a HLA-class I-restricted fashion. CTLs generated against these two novel peptides kill tumor cells of epithelial origin with greater efficacy than CTLs that were generated against the 263–271 nonamer peptide, but they do not kill normal epithelial tissue. These data demonstrate that T cells capable of reacting against epitopes of this tumor-associated antigen are not fully deleted from the repertoire, and the differential expression in tumor cells compared with normal cells suggests that this antigen might be used as a target for immunotherapy.
Ep-CAM-derived synthetic peptides, heteroclitic-modified peptides, and controls were tested for binding to human HLA-A*0201 in a T2 binding assay. The name, sequence, source, score of predicted binding, and half-life of binding as calculated by the method of Parker \(^4\) and as arbitrary units as calculated by the method of Rammensee \(^5\) and FI are shown. The binding of each peptide was analyzed at least three times, and the mean of all of the experiments performed is shown. A98-Id peptide sequence was obtained from the immunoglobulin heavy-chain sequence of a patient with plasma cell leukemia and is predicted to bind to HLA-B38.

### MATERIALS AND METHODS

#### Peptide Predictions
The protein sequence from Ep-CAM was reviewed for 9- and 10-mer peptides that could potentially bind to MHC class I molecules using a peptide-motif scoring system. \(^4\) A second analysis was performed using the computer-based prediction analysis of H. G. Rammensee, University of Tuebingen, Germany. \(^5\) Analysis was performed for HLA-A*0201 (a MHC class I allele expressed by nearly 50% of our cancer patients). We also examined the binding of peptides with amino acid substitutions that were predicted to enhance the binding affinity to this MHC class I allele.

#### Peptides
Peptides were generated as described previously \((11)\) and synthesized (Sigma Chemical Co.-Genosys Biotechnologies, Inc. The Woodlands, TX). Single amino acid substitutions at P9 and P10 respectively were made in those peptides that did not contain a valine at this position. Peptides that were synthesized and assayed are shown in Table 1. As a positive control for the HLA-A2 binding assay, a Mage-3-derived peptide (F271) was used. The immunoglobulin-derived peptide (A98-Id) was used as a negative control because this does not bind to HLA-A*0201. Peptides were dissolved in PBS at a peptide concentration of 2 mg/ml and stored at −70°C until use.

#### Peptide-binding assay
A previously described assay was used to determine whether synthetic peptides could bind to HLA-A*0201. \((11)\) Briefly, using TAP-deficient T2 hybridoma cells (American Type Culture Collection, Manassas, VA), FI is calculated by the mean fluorescence intensity MFI of HLA-A*0201 on T2 cells as determined by FACS analysis following the formula:

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FI = \frac{\text{MFI (T2 cells plus peptide)}}{\text{MFI (T2 cells without peptide)}} - 1
\]

#### Screening for Peptide-specific HLA-restricted T Cells
Using previously published methodology \((11)\), peptide-specific CTLs were generated in vitro using dendritic cells and CD40-activated B cells pulsed with Ep-CAM-derived peptides and were then tested for their ability to kill peptide-pulsed targets, normal lymphoid or epithelial cells, or tumor cells in an Ep-CAM-dependent fashion. Briefly, purified CD8\(^+\) T cells from six different HLA-A2\(^+\) normal donors were primed with irradiated peptide-pulsed autologous dendritic cells followed by weekly restimulations with autologous CD40-activated B cells. After three to four restimulations, >92% of cells in each culture were CD3\(^+\) lymphocytes, of which >90% were CD8\(^+\) and 0.4–3.2% were CD4\(^+\). Cultures contained <0.4% CD14\(^+\) and 3.0–4.2% CD56\(^+\). The cytotoxicity of generated CTL lines was assessed using peptide-pulsed CD40-activated HLA-A2-expressing normal B cells and tumor cell lines as targets in standard chromium release assays \((^{51}\text{Cr-release})\). MHC-restriction of lytic activity was tested against HLA-A2-negative tumors as well as by a blockade of the killing of HLA-A2-positive tumors using the anti-HLA-A2 mAb BB7.2 compared with the isotype-matched control mAb B5. Specific lysis of target cells is presented as percentage of specific \(^{51}\text{Cr-release}\), calculated from the formula: \((E - S \times T - S) 	imes 100\), where \(E\) is experimental \(^{51}\text{Cr-release}\), \(S\) is the spontaneous \(^{51}\text{Cr-release}\), and \(T\) is the total \(^{51}\text{Cr-release}\) by 1% Triton X-100.

#### RESULTS

**Binding Affinity of Native and Modified Ep-CAM Peptides.** Five peptides (four nonamer and one decaamer) derived from Ep-CAM and three heteroclitic modified peptides, that were predicted for binding according to the computer prediction programs, were synthesized and included in the peptide binding study. Three of the five native Ep-CAM-derived peptides (Ep-2, Ep-3, and Ep-5) strongly bound to HLA-A2 molecules with MFI ranging from 1.2 to 2.2 (Table 1). Binding affinity of all of the synthesized heteroclitic peptides toward MHC class I was greater than that of the parent peptide (FI ratios of heteroclitic:parent peptide were 1.25, 1.8, and 3.3). Low binding was observed for the two remaining Ep-CAM-derived peptides, Ep-1 and Ep-4. No binding was observed for the negative control peptide A98-Id (FI = 0). The Mage-3-derived and HLA-A2-restricted epitope exhibited strong binding affinity (MFI = 2.0).

**Specific CTL Reactivity against Autologous Targets.** T-cell lines were generated from three different normal HLA-A2\(^+\) donors against each of the Ep-Cam-derived peptides synthesized. CTLs generated against Ep-1 and Ep-4 did not kill the appropriate peptide-pulsed CD40-activated B cells. CTLs generated against peptides Ep-2 and Ep-3 specifically lysed autologous CD40-activated target B cells pulsed with the appropriate peptide but did not lyse the unpulsed CD40-activated target B cells (Fig. 1). CTLs that were generated against Ep-5, which had been previously shown immunogenic in the mouse transgenic model \((32)\), were significantly less effective at

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\(^4\) Internet address: http://bimas.dcmr.nih.gov/molbio/hla_bind/.

\(^5\) Internet address: http://134.2.96.221/Scripts/MHCserver.dll/EpPredict.htm.

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O. Gautschi, personal communication.
Table 2  Expression profile of tumor cell lines tested

<table>
<thead>
<tr>
<th>Tumor cell line</th>
<th>Organ</th>
<th>HLA-A2 expression</th>
<th>Ep-CAM expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW480</td>
<td>Colon</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HT29</td>
<td>Colon</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A549</td>
<td>Lung</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SW2</td>
<td>Lung</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Breast</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>T47D</td>
<td>Breast</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
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Fig. 1. Recognition of CD40-activated B-cell targets by cytotoxic T cells generated against each of the five Ep-CAM-derived peptides. T-cell lines were generated from three different normal HLA-A*0201 donors as described in "Materials and Methods" and were analyzed for cytotoxicity after four or five stimulations. Representative results are shown here for each peptide. Specific lysis is shown for each peptide for killing against CD40-B cells pulsed with peptide (■), CD40-B cells unpulsed (□), and pulsed with the irrelevant HLA-A*0201-binding peptide P271 derived from MAGE-3 (△).

Fig. 2. HLA-A2 restricted recognition of Ep-CAM-expressing malignant cells. Results are representative of experiments performed using six different HLA-A*0201 normal donors. A, cytotoxicity of Ep-2- and Ep-3-specific CTLs against colon cancer cell lines. Specific killing is demonstrated against the HLA-A2-positive cell line, SW480, by the Ep-2 (■)- and Ep-3 (●)-specific CTLs. No killing was observed against the parent peptide Ep-2 (□) or Ep-3 (○)-specific CTLs. Again, no killing was seen against unpulsed normal CD40-B cells (△). B, cytotoxicity of Ep-2- and Ep-3-specific CTLs against lung cancer cell lines. Specific killing is demonstrated against the HLA-A2-positive cell line A549 by the Ep-2 (■)- and Ep-3 (●)-specific CTLs. No killing was observed against the HLA-A2-negative but Ep-CAM-positive cell line SW2 by Ep-2 (□) or Ep-3 (○)-specific CTLs. Again, no killing was seen against unpulsed Ep-CAM-negative normal CD40-B cells (△). C, absence of specific cytotoxicity of Ep-2- and Ep-3-specific CTLs against breast cancer cell lines. No specific killing is demonstrated against the HLA-A2-negative breast cancer cell line MCF7 by the Ep-2 (■)- and Ep-3 (●)-specific CTLs. No killing was observed against the Ep-2 (□) or Ep-3 (○)-specific CTL. Again, no killing was seen against unpulsed normal CD40-B cells (△). D, absence of specific cytotoxicity of Ep-2- and Ep-3-specific CTLs against lung cancer cell lines. No specific killing is demonstrated against the HLA-A2-negative breast cancer cell line MCF7 by the Ep-2 (■)- and Ep-3 (●)-specific CTLs. No killing was observed against the Ep-2 (□) or Ep-3 (○)-specific CTL. Again, no killing was seen against unpulsed normal CD40-B cells (△).

DISCUSSION

Antibody-directed therapy with murine mAbs targeting the Ep-CAM antigen has resulted in improved survival of patients (30) and might be optimized by novel, fully humanized mAbs that lack the induction of human antihouse antibodies during therapy (39). The wide applicability and limited toxicity of these antibody-based studies and reactivity of T cells against one epitope (33) indicates the potential of this antigen for immunotherapy. We now demonstrate in a human cellular system that novel immunogenic Ep-CAM-derived epitopes can also function as a target for specific CD8+ cytotoxic T-cell response. Using a bioinformatics approach, we synthesized five native and three heteroclitic peptides with predicted binding to HLA-A*0201. Three of the five synthesized native peptides and all of the heteroclitic peptides strongly bound to HLA-A*0201. When an efficient T-cell screening system was used, three native peptides and all of the heteroclitic peptides tested induced peptide-specific CD8+ T-cell responses in an antigen-specific and MHC-restricted fashion in vitro. Ep-CAM is commonly expressed on the surface of human epithelial malignancies, is not expressed on normal squamous epithelial tissue (40), and is expressed on the basolateral surface of normal epithelial tissue (41). The reason why the CTLs killed in vitro the
tumor cells that express Ep-CAM yet did not kill normal epithelial tissue that also expresses Ep-CAM is not clear. In vivo, there is an altered expression pattern of Ep-CAM on tumor cells of epithelial origin as compared with normal epithelium, which may allow cytotoxic T-cell lines generated with these peptides to recognize and lyse Ep-CAM-expressing human cancer cell lines preferentially, compared with normal epithelial cells. Cytotoxicity was also demonstrated against human colon and lung carcinomas. Killing occurred in a HLA-restricted fashion as demonstrated by the antibody-blocking studies and lack of lytic activity against HLA-A2-negative but Ep-CAM-expressing tumors.

We also demonstrated that an intact T-cell repertoire against several Ep-CAM-derived epitopes exists in humans. Moreover, stimulation of HLA-A2+ peripheral blood mononuclear cells from healthy donors with immunodominant Ep-CAM-derived peptides efficiently promotes the expansion of specific CTLs. It should, however, be stressed that we have demonstrated here not that the Ep-Cam-expressing cell lines are actually processing and presenting these peptide epitopes but that the T-cell lines could react with related or cross-reacting peptides.

Our observations suggest that Ep-CAM derived peptides might also serve as a basis for CTL mediated therapy in patients with Ep-CAM-expressing tumors, such as demonstrated here for adenocarcinomas of colon, lung, and breast. Recent studies have demonstrated the presence of CTLs against Ep-CAM in cancer-bearing patients, which indicates that tolerance is not induced against this tumor antigen. (33).

Previous work has demonstrated that heteroclitic peptides can elicit efficient T-cell responses and increased cytotoxicity against tumor cells (10, 12). Of particular note, although CTLs could be generated against peptide-pulsed antigen-presenting cells that killed colon and lung cancer cell lines, only CTLs generated against the heteroclitic peptides were capable of killing the Ep-CAM-expressing breast cancer MCF-7, which was not lysed by any of the CTLs generated with native peptides (possibly because of the low HLA-A2 expression observed in this cell line). Lytic activity of the CTLs generated against the heteroclitic peptides was significantly enhanced against all of the HLA-A2-expressing cancer cell lines tested; and heteroclitic peptides can elicit T-cell responses not only against the altered peptides but also against the native peptide, either when presented by pulsed antigen-presenting cells or, more importantly, when the antigen is presented by the tumor cell itself. Heteroclitic peptides might, therefore, increase the efficiency of tumor-cell killing when low presentation of MHC-peptide complexes on tumor cells (42) or suppression of immune cells (43) causes an inadequate immune response.

Together, the wide expression of Ep-CAM in epithelial tumors and the finding that novel immunogenic peptides can elicit an effective immune response against tumor cells but not normal epithelial cells suggests that these epitopes represent an antigen that may indeed be suitable for T-cell-mediated immunotherapy. For such an approach to be successful, a sufficient T-cell repertoire in the cancer-bearing patient will be required to elicit a cytotoxic T-cell response against the tumor. We have been able to readily expand peptide-specific CTLs and to demonstrate precursor CTLs derived from blood lymphocytes against the immunogenic peptide Ep-2, and its heteroclitic variant, in the majority of 11 tested HLA-A2+ patients with Ep-CAM-expressing cancer of colon and lung. (33) On the basis of these findings, we believe that the use of heteroclitic peptide-pulsed antigen-presenting cells may be an attractive strategy for vaccination and adoptive T-cell immunotherapy approaches against Ep-CAM-expressing tumors.

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REFERENCES


A. Trojan, unpublished observation.
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