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Use of Two Predictive Algorithms of the World Wide Web for the Identification of Tumor-reactive T-Cell Epitopes[1]

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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 immune therapies 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 that are costly and labor-intensive. We report here the use of two computer-based prediction algorithms, 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.[3] CEA is a M_r 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 on 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.[4] This computer algorithm ranks potential MHC binders according to the predictive half-time disassociation of peptide/MHC complexes. The second algorithm, "SYFPEITHI,"[5], 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: $CEA9_{185/363/541}$ (LPVSPRLQL), $CEA9_{442}$ (NPPAQYSWL), and $CEA9_{632}$ (IPQQHTQVL). These peptides were synthesized according to standard solid-phase synthesis methods using Applied Biosystems apparatus and were purified by high-performance liquid chromatography. The purity

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[3] The abbreviations used are: TAA, tumor-associated antigen; CEA, carcinoembryonic antigen; DC, dendritic cell; IL, interleukin; rIL, recombinant IL; BGP, biliary glycoprotein; NCA, nonspecific cross-reactive antigen.

[4] Internet address: http://bimas.dcrt.nih.gov/molbio/ken_parker_combofrom.

[5] Internet address: http://134.2.96.221/scripts/hlaserver.dll/home.htm.

(>95%) and identity of peptides were determined by analytical high-performance liquid chromatography and mass spectrometry analysis. Peptides were dissolved at 10 mg/ml in DMSO containing 0.1% trifluoroacetic acid and were aliquoted in small volumes to be maintained frozen at −20°C until further use.

Cell Lines. 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 erithroleukemia 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 624mel 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 cytotoxicity 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 0.25×10^5 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 (28) 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 ^{51}Cr release assay as described (29). Peptide-pulsed targets were prepared by incubating JY cells with 10 μg/ml peptides at 37C° overnight. Adherent tumor cells were removed from culture flask with trypsin-EDTA immediately before ^{51}Cr-labeling. Target cells were labeled with 300 μCi [^{51}Cr]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:

$$\frac{\text{cpm of the test sample} - \text{cpm of spontaneous release}}{\text{cpm of the maximal release} - \text{cpm of spontaneous release}} \times 100$$

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 using 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:

$$\frac{\%\text{ specific lysis without inhibitors} - \%\text{ specific lysis with inhibitors}}{\%\text{ specific lysis without inhibitors}} \times 100$$

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 (LPVSPRLQL) 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 $CEA9_{632}$ 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

Table 1 *List of candidate HLA-B7 CTL epitopes for CEA*[a]

Antigen	Sequence	Position of first residue	BIMAS score[b]	SYFPEITHI score[c]	CTL activity[d]
Predicted epitopes					
CEA	PPTTAKLTI	30	0.8	18	NT[e]
CEA	GPAYSGREI	92	8	18	NT
CEA	YPELPKPSI	141	3.6	20	NT
CEA	**LPVSPRLQL**	**185/363/541**	**180**	**26**	**0/96**
CEA	**NPPAQYSWL**	**442**	**80**	**21**	**0/96**
CEA	PPAQYSWLI	443	0.8	17	NT
CEA	**IPQQHTQVL**	**632**	**80**	**23**	**3/96**
Known epitopes					
EBNA-6[f]	QPRAPIRPI	881	120	23	NT
EBNA-3[f]	RPPIFIRRL	247	80	21	NT
RU2-AS[g]	LPRWPPPQL	ND	1200	26	NT
iCE (+1ORF)[h]	SPRWWPTCL	ND	800	23	NT

[a] In bold type: potential *HLA-B7*-restricted CTL epitopes for CEA.
[b] Algorithm score obtained using the computer program developed by Parker *et al.* (20).
[c] Algorithm score obtained using the computer program developed by Rammensee *et al.* (21).
[d] CTL activity is the number of positive CTL cultures in a total of 96 from two blood donors.
[e] NT, not tested; ND, not determined.
[f] CTL epitope described in 22, 23.
[g] CTL epitope described in 24.
[h] CTL epitope described in 25.

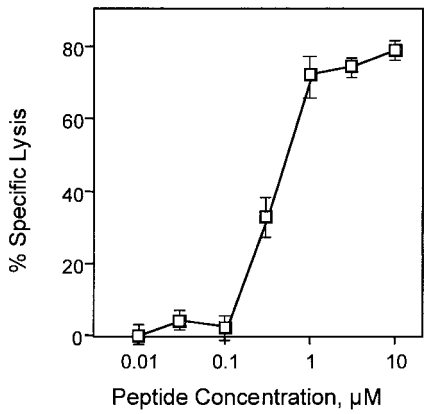

Fig. 1. Peptide dose-response curve of CEA-specific CTLs. Radiolabeled JY cells were incubated with various concentrations of peptide $CEA9_{632}$ for 30 min, and CTLs were added at a final E:T ratio of 10:1. Cytotoxicity was determined after 4 h as described in "Materials and Methods." Results are expressed as mean ± SE of triplicate samples.

responding to <1 μM of peptide $CEA9_{632}$ (example presented in Fig. 1), which suggests that their affinity for antigen may be sufficiently high to enable them to recognize CEA+ tumor cells.

Antitumor Reactivity of CEA-specific CTLs. The results in Fig. 2 demonstrate that CTL clones derived from the two *HLA-B7* individuals were very effective in killing peptide-pulsed JY target cells as well as two CEA+ *HLA-B7* tumor cell lines, one a colon carcinoma (SW403) and the other an immortalized breast cell line (HBL-100). Interestingly, the level of cytotoxicity toward the SW403 cells obtained with one of the clones was similar to the one obtained toward peptide-pulsed JY cells (Fig. 2*B*). Both of the clones did not kill peptide unpulsed JY cells or the 624mel tumor cell line (*HLA-B7*+, CEA− melanoma), which demonstrates that the recognition of CEA CTL epitope represented by peptide $CEA9_{632}$ is antigen-specific. These results also indicate that the CEA-producing cell lines, SW403 and HBL-100, effectively process and present peptide $CEA9_{632}$ in the context of the *HLA-B7* allele.

HLA Restriction and Antigen-Specificity Analysis. To confirm that the recognition of the peptide $CEA9_{632}$ by the CTL clones was MHC 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. 3*A*) and HBL-100 (Fig. 3*B*) by CEA-specific CTL (the clone shown in Fig. 2*B*) 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 $CEA9_{632}$ was corroborated in a cold-target inhibition assay in which unlabeled (cold) JY cells that were pulsed or not with peptide $CEA9_{632}$ 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. 4*A*) and HBL-100 (Fig. 4*B*), was inhibited significantly by the $CEA9_{632}$-pulsed JY cells but not by the unpulsed JY cells. These results demonstrate that $CEA9_{632}$-specific CTL can recognize the naturally processed epitope expressed on the surface of CEA+ tumor cells.

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

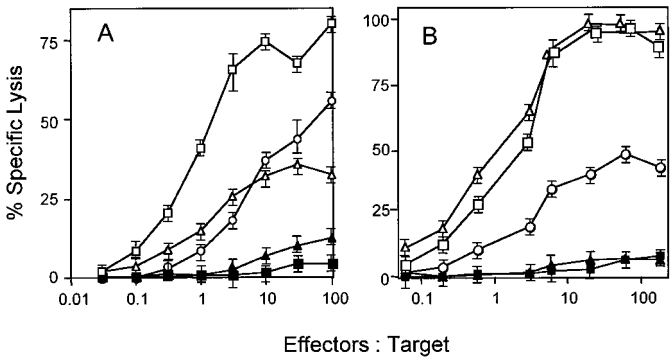

Fig. 2. CTLs induced with peptide $CEA9_{632}$ can recognize tumors expressing CEA. Two different CTL clones isolated from two normal *HLA-B7*+ volunteers (*A* and *B*) were tested for their capacity to recognize tumor cells expressing CEA. Cytotoxicity was measured in a 4-h ^{51}Cr release assay at various E:T ratios against the following target cells: □, JY pulsed with $CEA9_{632}$; ■, JY without peptide; △, SW403 (colon cancer, B7+, CEA+); ○, HBL100 (breast, B7+, CEA+); ▲, 624mel (melanoma, B7+, CEA−). Results are expressed as mean ± SE of triplicate samples.

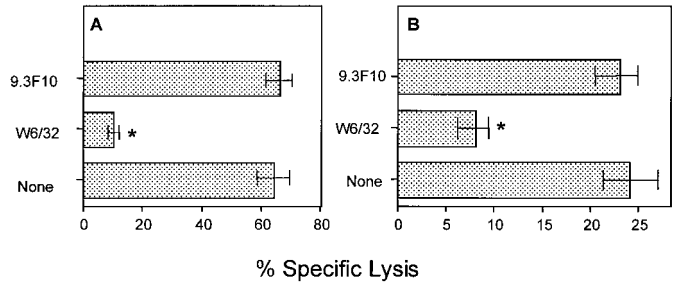

Fig. 3. CEA-specific CTLs recognize antigen presented by MHC class I molecules. Monoclonal antibodies specific for MHC class I molecules (W6/32), but not antibodies reactive with MHC class II molecules (9.3F10), inhibited the lysis of the colon cancer cell line SW403 (*A*) or the breast cell line HBL100 (*B*) by a $CEA9_{632}$-specific CTL clone. Both antibodies were tested at 10 μg/ml, and cytotoxicity was determined at an E:T ratio of 3:1. Results are expressed as mean ± SE of triplicate samples. *, $P < 0.01$.

possibility of using hypothetical analyses that are available in the public domain to predict for MHC-binding peptides as candidates for CTL epitopes. Nevertheless, one must be aware that any predictive algorithm will not be 100% accurate, and the possibility may exist that all of the high-scoring peptides may not actually bind to the MHC molecule. For this reason, it may be necessary to test several of the predicted epitopes for their ability to trigger CTL responses to obtain at least one true CTL epitope. For the present studies, we selected CEA as the TAA and *HLA-B*0702* as the MHC-restricting allele for the following three reasons: (*a*) CEA is an ideal TAA because it is found overexpressed in a great variety of solid tumors (14, 15) and is a large molecule (702 residues), which increases the probability of identifying T-cell peptide epitopes: (*b*) several CEA CTL epitopes restricted by *HLA-A2*, *-A3*, and *-A24* have been described (16–19), which indicates that the choice of this TAA for T-cell-based immunotherapy is reasonable; and (*c*) the identification of CEA CTL epitopes restricted by *HLA-B7*, another frequently found MHC class-I allele [and the prototype member of the *HLA-B7* superfamily (30)] would significantly extend population coverage for an immune-based approach to treat CEA+ tumors. By combining two computer-based algorithms, we narrowed the list of potential *HLA-B*0702*−restricted CTL epitopes for CEA to three candidate peptides. Subsequently, using an *in vitro* CTL vaccination procedure that was developed in our laboratory (28), we tested the capacity of the three candidate peptides to elicit tumor-reactive CTL.

Following this strategy, we have successfully identified one *HLA-B7*-restricted epitope from CEA. The results demonstrate that peptide $CEA9_{632}$- 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 $CEA9_{632}$ 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 $CEA9_{632}$ 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 $CEA9_{185/363/541}$ and $CEA9_{442}$ 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 $CEA9_{185/363/541}$ and $CEA9_{442}$ 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 $CEA9_{632}$ (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 $CEA9_{185/363/541}$ and $CEA9_{442}$ will 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 $CEA9_{632}$ 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 $CEA9_{632}$ 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 $CEA9_{632}$ (IPQQHTQVL) score as "favorable" or "neutral" using the B7-like supermotif described by Sidney *et al.* (30), with the exception of the Q_7, which scores as "deleterious" for all of the alleles including *B*0702*. Nonetheless, it will be necessary to demonstrate that peptide $CEA9_{632}$ 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 $CEA9_{632}$ 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

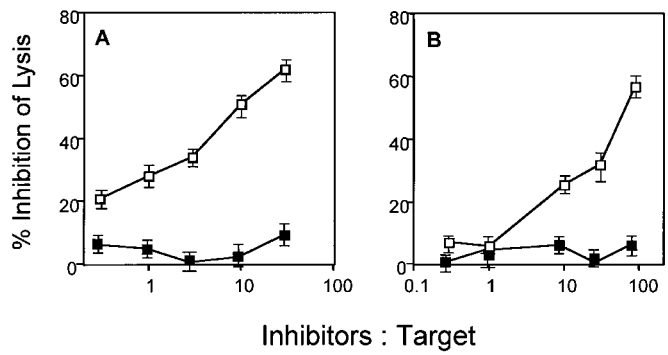

Fig. 4. Antigen specificity of $CEA9_{632}$-specific CTL clones demonstrated by cold-target inhibition assays. CEA+, *HLA-B7*+ tumor cell lines SW403 (*A*) and HBL100 (*B*) were labeled with ^{51}Cr and mixed at various inhibitors to target ratios with the following cold (unlabeled) targets: JY pulsed with 10 μg/ml peptide $CEA9_{632}$ (□) and without peptide (■). By using $CEA9_{632}$-specific CTL clones as effectors, cytotoxicity was measured at an E:T ratio of 10:1. Results are presented as the percentage inhibition means (± SE) of specific lysis calculated from triplicate samples as described in "Materials and Methods."

tumor models (43–45). Either approach using peptide $CEA9_{632}$ as immunogen for CTLs is worth considering for future clinical studies in *HLA-B7* patients with colon, lung, or breast tumors expressing CEA. Because CD4+ helper T lymphocytes play a critical role in the establishment and long-term maintenance of antigen-specific CTLs, any immunological approach to treat tumors should seek also to stimulate tumor antigen-reactive helper T cells in addition to CTLs. For this reason, our laboratory is actively seeking to identify MHC class II-restricted T-helper epitopes from CEA.

REFERENCES

1. Rooney, C. M., Heslop, H. E., and Brenner, M. K. EBV specific CTL: a model for immune therapy. Vox Sang. *74* (Suppl. 2)*:* 497–498, 1998.
2. Coulie, P. G. Human tumour antigens recognized by T cells: new perspectives for anti-cancer vaccines? Mol. Med. Today, *3:* 261–268, 1997.
3. Kawakami, Y., and Rosenberg, S. A. Immunobiology of human melanoma antigens MART-1 and gp100 and their use for immuno-gene therapy. Int. Rev. Immunol., *14:* 173–192, 1997.
4. Toes, R. E., Offringa, R., Feltkamp, M. C., Visseren, M. J., Schoenberger, S. P., Melief, C. J., and Kast, W. M. Tumor rejection antigens and tumor specific cytotoxic T lymphocytes. Behring Inst. Mitt., 72–86, 1994.
5. Boon, T., Cerottini, J. C., Van den Eynde, B., van der Bruggen, P., and Van Pel, A. Tumor antigens recognized by T lymphocytes. Annu. Rev. Immunol., *12:* 337–365, 1994.
6. Heslop, H. E., and Rooney, C. M. Adoptive cellular immunotherapy for EBV lymphoproliferative disease. Immunol. Rev., *157:* 217–222, 1997.
7. Nestle, F. O., Alijagic, S., Gilliet, M., Sun, Y., Grabbe, S., Dummer, R., Burg, G., and Schadendorf, D. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat. Med., *4:* 328–332, 1998.
8. Rosenberg, S. A., Yang, J. C., Schwartzentruber, D. J., Hwu, P., Marincola, F. M., Topalian, S. L., Restifo, N. P., Dudley, M. E., Schwarz, S. L., Spiess, P. J., Wunderlich, J. R., Parkhurst, M. R., Kawakami, Y., Seipp, C. A., Einhorn, J. H., and White, D. E. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat. Med., *4:* 321–327, 1998.
9. Thurner, B., Haendle, I., Roder, C., Dieckmann, D., Keikavoussi, P., Jonuleit, H., Bender, A., Maczek, C., Schreiner, D., von Den Driesch, P., Brocker, E. B., Steinman, R. M., Enk, A., Kampgen, E., and Schuler, G. Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. J. Exp. Med., *190:* 1669–1678, 1999.
10. Rosenberg, S. A., Packard, B. S., Aebersold, P. M., Solomon, D., Topalian, S. L., Toy, S. T., Simon, P., Lotze, M. T., Yang, J. C., Seipp, C. A., Simpson, C., Carter, C., Rock, S., Schwartzentruber, D., Wei, J. P., and White, D. E. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A report. N. Engl. J. Med., *319:* 1676–1680, 1988.
11. Germain, R. N., and Margulies, D. H. The biochemistry and cell biology of antigen processing and presentation. Annu. Rev. Immunol., *11:* 403–450, 1993.
12. York, I. A., and Rock, K. L. Antigen processing and presentation by the class I major histocompatibility complex. Annu. Rev. Immunol., *14:* 369–396, 1996.
13. Rammensee, H. G., Rotzschke, O., and Falk, K. MHC class I-restricted antigen processing: lessons from natural ligands. Chem. Immunol., *57:* 113–133, 1993.
14. Shively, J., and Beatty, J. CEA-related antigens: molecular biological and clinical significance. CRC Crit. Rev. Oncol. Hematol., *2:* 355–399, 1985.
15. Thompson, J., Grunert, F., and Zimmerman, W. CEA gene family: molecular biology and clinical perspectives. J. Clin. Lab. Anal., *5:* 344–366, 1991.
16. Tsang, K. Y., Zaremba, S., Nieroda, C. A., Zhu, M. Z., Hamilton, J. M., and Schlom, J. Generation of human cytotoxic T cells specific for human carcinoembryonic antigen epitopes from patients immunized with recombinant vaccinia-CEA vaccine. J. Natl. Cancer Inst., *87:* 982–990, 1995.
17. Nukaya, I., Yasumoto, M., Iwasaki, T., Ideno, M., Sette, A., Celis, E., Takesako, K., and Kato, I. Identification of HLA-A24 epitope peptides of carcinoembryonic antigen which induce tumor-reactive cytotoxic T lymphocyte. Int. J. Cancer, *80:* 92–97, 1999.
18. Kawashima, I., Tsai, V., Southwood, S., Takesako, K., Sette, A., and Celis, E. Identification of HLA-A3-restricted cytotoxic T lymphocyte epitopes from carcinoembryonic antigen and HER-2/*neu* by primary *in vitro* immunization with peptide-pulsed dendritic cells. Cancer Res., *59:* 431–435, 1999.
19. Kawashima, I., Hudson, S., Tsai, V., Southwood, S., Takesako, K., Appella, E., Sette, A., and Celis, E. The multi-epitope approach for immunotherapy for cancer: identification of several CTL epitopes from various tumor-associated antigens expressed on solid epithelial tumors. Hum. Immunol., *59:* 1–14, 1998.
20. Parker, K. C., Bednarek, M. A., and Coligan, J. E. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J. Immunol., *152:* 163–175, 1994.
21. Rammensee, H., Bachmann, J., Emmerich, N. P., Bachor, O. A., and Stevanovic, S. SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics, *50:* 213–219, 1999.
22. Maier, R., Falk, K., Rotzschke, O., Maier, B., Gnau, V., Stevanovic, S., Jung, G., Rammensee, H. G., and Meyerhans, A. Peptide motifs of HLA-A3, -A24, and -B7 molecules as determined by pool sequencing. Immunogenetics, *40:* 306–308, 1994.
23. Hill, A. B., Lee, S. P., Haurum, J. S., Murray, N., Yao, Q. Y., Rowe, M., Signoret, N., Rickinson, A. B., and McMichael, A. J. Class I major histocompatibility complex-restricted cytotoxic T lymphocytes specific for Epstein-Barr virus (EBV)-transformed B lymphoblastoid cell lines against which they were raised. J. Exp. Med., *181:* 2221–2228, 1995.
24. Ronsin, C., Chung-Scott, V., Poullion, I., Aknouche, N., Gaudin, C., and Triebel, F. A non-AUG-defined alternative open reading frame of the intestinal carboxyl esterase mRNA generates an epitope recognized by renal cell carcinoma-reactive tumor-infiltrating lymphocytes *in situ*. J. Immunol., *163:* 483–490, 1999.
25. Van Den Eynde, B. J., Gaugler, B., Probst-Kepper, M., Michaux, L., Devuyst, O., Lorge, F., Weynants, P., and Boon, T. A new antigen recognized by cytolytic T lymphocytes on a human kidney tumor results from reverse strand transcription. J. Exp. Med., *190:* 1793–1800, 1999.
26. Romani, N., Gruner, S., Brang, D., Kampgen, E., Lenz, A., Trockenbacker, B., Konwalkinka, G., Fritsch, P. O., Steinman, R. M., and Schuler, G. Proliferating dendritic cell progenitors in human blood. J. Exp. Med., *180:* 83–93, 1994.
27. Sallusto, F., and Lanzavecchia, A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α, J. Exp. Med. *179:* 1109–1118, 1994.
28. Tsai, V., Kawashima, I., Keogh, E., Daly, K., Sette, A., and Celis, E. *In vitro* immunization and expansion of antigen-specific cytotoxic T lymphocytes for adoptive immunotherapy using peptide-pulsed dendritic cells. Crit. Rev. Immunol., *18:* 65–75, 1998.
29. Celis, E., Fikes, J., Wentworth, P., Sidney, J., Southwood, S., Maewal, A., Del Guercio, M. F., Sette, A., and Livingston, B. Identification of potential CTL epitopes of tumor-associated antigen MAGE-1 for five common *HLA-A* alleles. Mol. Immunol., *31:* 1423–1430, 1994.
30. Sidney, J., Southwood, S., del Guercio, M. F., Grey, H. M., Chesnut, R. W., Kubo, R. T., and Sette, A. Specificity and degeneracy in peptide binding to *HLA-B7*-like class I molecules. J. Immunol., *157:* 3480–3490, 1996.
31. Culmann, B., Gomard, E., Kieny, M. P., Guy, B., Dreyfus, F., Saimot, A. G., Sereni, D., Sicard, D., and Levy, J. P. Six epitopes reacting with human cytotoxic CD8+ T cells in the central region of the HIV-1 NEF protein. J. Immunol., *146:* 1560–1505, 1991.
32. Wang, W., Meadows, L. R., den Haan, J. M., Sherman, N. E., Chen, Y., Blokland, E., Shabanowitz, J., Agulnik, A. I., Hendrickson, R. C., Bishop, C. E., Hunt, D. F., Goulmy, E., and Engelhard, V. H. Human H-Y: a male-specific histocompatibility antigen derived from the SMCY protein. Science (Washington DC), *269:* 1588–1590, 1995.
33. Celis, E., Tsai, V., Crimi, C., DeMars, R., Wentworth, P. A., Chesnut, R. W., Grey, H. M., Sette, A., and Serra, H. M. Induction of anti-tumor cytotoxic T lymphocytes in normal humans using primary cultures and synthetic peptide epitopes. Proc. Natl. Acad. Sci. USA, *91:* 2105–2109, 1994.
34. Tsai, V., Southwood, S., Sidney, J., Sakaguchi, K., Kawakami, Y., Appella, E., Sette, A., and Celis, E. Identification of subdominant CTL epitopes of the gp100 melanoma-associated tumor antigen by primary *in vitro* immunization with peptide-pulsed dendritic cells. J. Immunol., *158:* 1796–1802, 1997.
35. Tanaka, F., Fujie, T., Tahara, K., Mori, M., Takesako, K., Sette, A., Celis, E., and Akiyoshi, T. Induction of antitumor cytotoxic T lymphocytes with a MAGE-3-encoded synthetic peptide presented by human leukocytes antigen-A24. Cancer Res., *57:* 4465–4468, 1997.
36. Kawashima, I., Tsai, V., Southwood, S., Takesako, K., Celis, E., and Sette, A. Identification of gp100-derived, melanoma-specific cytotoxic T- lymphocyte epitopes restricted by HLA-A3 supertype molecules by primary *in vitro* immunization with peptide-pulsed dendritic cells. Int. J. Cancer, *78:* 518–524, 1998.
37. Sette, A., Sidney, J., del Guercio, M. F., Southwood, S., Ruppert, J., Dahlberg, C., Grey, H. M., and Kubo, R. T. Peptide binding to the most frequent *HLA-A* class I alleles measured by quantitative molecular binding assays. Mol. Immunol., *31:* 813–822, 1994.
38. Zugel, U., Wang, R., Shih, G., Sette, A., Alexander, J., and Grey, H. M. Termination of peripheral tolerance to a T cell epitope by heteroclitic antigen analogues. J. Immunol., *161:* 1705–1709, 1998.
39. Zaks, T. Z., and Rosenberg, S. A. Immunization with a peptide epitope (p369–377) from HER-2/neu leads to peptide-specific cytotoxic T lymphocytes that fail to recognize HER-2/neu+ tumors. Cancer Res., *58:* 4902–4908, 1998.
40. Weber, J. S., Hua, F. L., Spears, L., Marty, V., Kuniyoshi, C., and Celis, E. A Phase I trial of an HLA-A1 restricted MAGE-3 epitope peptide with incomplete Freund's adjuvant in patients with resected high-risk melanoma. J. Immunother., *22:* 431–440, 1999.
41. Marchand, M., Weynants, P., Rankin, E., Arienti, F., Belli, F., Parmiani, G., Cascinelli, N., Bourlond, A., Vanwijck, R., Humblet, Y., Canon, J-L., Laurent, C., Naeyaert, J-M., Plagne, R., Daraemaeker, R., Knuth, A., Jager, E., Brasseur, F., Herman, J., Coulie, P. G., and Boon, T. Tumor regression responses in melanoma patients treated with a peptide encoded by gene MAGE-3. Int. J. Cancer, *63:* 883–885, 1995.
42. Jager, E., Ringhoffer, M., Dienes, H. P., Arand, M., Karbach, J., Jager, D., Ilsemann, C., Hagedorn, M., Oesch, F., and Knuth, A. Granulocyte-macrophage-colony-stimulating factor enhances immune responses to melanoma-associated peptides *in vivo*. Int. J. Cancer, *67:* 54–62, 1996.
43. Porgador, A., Snyder, D., and Gilboa, E. Induction of antitumor immunity using bone marrow-generated dendritic cells. J. Immunol., *156:* 2918–2926, 1996.
44. Mayordomo, J. I., Zorina, T., Storkus, W. J., Celluzzi, C., Falo, L., Kast, W. M., Ildstad, S. T., DeLeo, A. B., and Lotze, M. T. Bone marrow-derived dendritic cells pulsed with tumor peptides elicit protective and therapeutic antitumor immunity. Nat. Med., *1:* 1297–1303, 1995.
45. Ribas, A., Butterfield, L. H., Hu, B., Dissette, V. B., Chen, A. Y., Koh, A., Amarnani, S. N., Glaspy, J. A., McBride, W. H., and Economou, J. S. Generation of T-cell immunity to a murine melanoma using MART-1-engineered dendritic cells. J. Immunother., *23:* 59–66, 2000.

Use of Two Predictive Algorithms of the World Wide Web for the Identification of Tumor-reactive T-Cell Epitopes

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