Recognition of Carcinoembryonic Antigen Peptide and Heteroclitic Peptide by Peripheral Blood T Lymphocytes

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Summary: The carcinoembryonic antigen (CEA)-derived peptide CAP1 and heteroclitic peptide CAP1-6D are stimulators of HLA-A*A0201 restricted CEA-specific T cells in vivo and in vitro. The goal of this study was to evaluate differences in T cell responses to peptide and modified peptide antigens from CEA. The heterogeneity of responses among individuals is potentially important for the design of future CEA-directed immunotherapy trials. Peripheral blood mononuclear cells from blood donors were stimulated with peptide, IL-2, and IL-7. Weekly, microcultures were restimulated with irradiated, autologous peptide-loaded peripheral blood mononuclear cells and expanded in IL-2. Established T cell lines were tested by cytokine release assays using peptide-loaded T2 targets. T cell avidity was measured by cytokine release using targets expressing diminishing concentrations of peptide. Fine specificities were measured using targets loaded with alanine-substituted CAP1 peptide. Tumor recognition was measured using HLA-A*A0201/CAP1-transduced COS tumor targets. Varied responses to CAP1 and CAP1-6D were seen among individuals. The immunogenicity of CAP1 or CAP1-6D was donor dependent. Many T cells recognized one peptide but did not cross-recognize the altered peptide. The avidities of T cell lines were moderate to low, and fine specificities were consistent with a narrow antigen-specific repertoire. CAP1-6D-based immune therapy may not be optimal in some patients with CAP1-specific precursors. The T cell repertoire may be a central contributor to the limited responses seen with CEA-directed immunotherapy to date. Treatment strategies designed to alter or expand the T cell repertoire against CEA should be considered for trials.

Key Words: T cell, carcinoembryonic antigen, T cell avidity, heteroclitic antigen

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T lymphocytes are capable of recognizing antigenic peptides derived from normal proteins that are expressed on many types of cancer.[1] The interaction of the T cell receptor (TCR) with the major histocompatibility complex (MHC)-peptide complex is highly specific, and signaling by the T cell may vary with slight alterations of the MHC-peptide conformation.[2] When peptide antigens are altered through changes in the amino acid sequence without abrogating T cell recognition, heteroclitic peptide antigens are created.[3] Many heteroclitic peptides have been described for tumor-associated antigens that either improve T cell recognition by effecting better peptide to MHC binding at the primary anchor residues,[4–10] or that enhance TCR binding to the peptide-MHC complex.[11–16] The potential for heteroclitic peptides to break self-antigen tolerance has led to their rapid adoption for clinical immunotherapy trials. The use of heteroclitic antigens is particularly appealing for tumors that express poorly immunogenic antigens, such as the carcinoembryonic antigen (CEA) expressed on many common adenocarcinomas.[17,18]

CEA is a protein antigen shared among several common human adenocarcinomas that are leading causes of cancer death.[19–23] As such, it is an attractive target for investigators desiring to expand the scope of immunotherapy to the treatment of tumors other than melanoma and renal cell carcinoma. The immunodominant HLA-A*A0201 restricted peptide epitope from CEA is a nonapeptide beginning at residue 605 of the translated CEA gene, or at residue 571 beyond a 34 amino acid leader sequence (CEA:605–613, or CEA:571–579). It is most commonly referred to as CAP1.[24] A heteroclitic peptide analog to CAP1 has been generated by substituting aspartic acid for asparagine at the sixth amino acid position (CAP1-6D). The CAP1-6D peptide was identified from rationally designed heteroclitic peptides by its ability to stimulate a T cell line generated from a patient treated with a CEA-based tumor vaccine.[18] The 6D substitution did not affect peptide binding to HLA-A*A0201, but was more readily recognized than CAP1 by this CAP1-reactive T cell line. Since the description of the CAP1-6D epitope, clinical trials have been performed to evaluate its ability to immunize patients with CEA-bearing tumors. In at least one such trial, tumor regression was seen in treated cancer patients, and clinical responses were correlated with an increase in CAP1 tetramer binding by T cells after vaccination.[17]

Whether CAP1-6D will prove a heteroclitic antigen for the majority of CAP1-reactive T cell clones is not clear. Few CAP1-6D reactive lines have been established

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for testing, perhaps due to the low frequency of CAP1-reactive cells in the circulation and the difficulty establishing clones in vitro. The utility of CAP1-6D for immunotherapy will be determined by the prevalence of CAP1 and CAP1-6D–specific cells in an individual's repertoire, the cross-recognition of CAP1 by CAP1-6D–specific cells, and the avidity of CAP1-6D reactive cells for the native antigen CAP1. In an individual with a limited CAP1-6D reactive repertoire or poor cross-reactivity of these cells for CAP1, immunotherapy with heteroclitic peptide will be of no benefit. For such a patient, other strategies, such as abrogating suppression or inducing homeostatic proliferation, are potentially superior to the use of heteroclitic antigen for breaking tolerance to self-antigen.[25,26]

We investigated the prevalence of expandable CAP1 and CAP1-6D reactive T cells populations in blood donors. Using a semiquantitative in vitro stimulation assay, we identified a broad range in the prevalence of antigen-specific T cells in the peripheral blood. In individuals who had a readily expandable CAP1 or CAP1-6D reactive T cell population, the cross-reactivity of these cells for heteroclitic antigen was investigated. The relative avidity of these cells was assessed and the breadth of the antigen-specific repertoire in each individual was investigated by examining several reactive populations using a panel of alanine-substituted CAP1 peptides. In sum, expandable CAP1 or CAP1-6D reactive T cells were not found in all individuals. T cell populations were identified that recognized CAP1 but were unable to be stimulated by CAP1-6D, as were populations that were readily expanded by CAP1-6D stimulation but were unable to recognize CAP1. CAP1-reactive populations had modest functional avidity in culture and were from narrow repertoires. Taken together, these results suggest a subpopulation of individuals exist for whom CEA-directed therapy with CAP1-6D has no advantage over therapy with the native CAP1 peptide.

METHODS

Cells and Cell Lines

Tumor infiltrating lymphocyte culture TIL 1520 was established from a melanoma patient at the Surgery Branch, NCI and maintained in AIM V human complete medium consisting of AIM V (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% heat-inactivated pooled human AB serum (Valley Biomedical, Winchester, VA), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (Mediatech, Herndon, VA). TIL cultures were supplemented with 6000 IU/mL rh IL-2 (Chiron, Emeryville, CA). The HLA-A*A0201-expressing human T lymphoblast-B lymphoblast hybrid cell line, T2 (ATCC, Manassas, VA) was used as a T cell target after loading with CEA or control peptide. The T2 line does not present endogenously processed antigen, but exogenous peptides are presented with MHC class I molecules on the surface of these cells. T2 cells were maintained in RPMI 1640 complete medium consisting of RPMI 1640 (Mediatech) supplemented with 10% fetal bovine serum (Invitrogen Life Technologies), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine.

CAP1 expressing tumor targets were created by transducing the COS and COS-A2 (COS cells retrovirally transduced to express HLA-A*A0201) cell lines with a retroviral construct capable of expressing the CAP1 epitope from human CEA. Complementary synthetic oligonucleotides were synthesized (5′-GGCCCGCG CAGGCAGCATGTACCTTT-3′, 5′-GGCCTCACTA GAGGTTGAGGTTCGCT-3′) containing the DNA sequence for the CAP1 peptide epitope preceded by the Kozak[27] consensus sequence and an ATG start codon. The epitope minigene was ligated into a SAMEN-based retroviral vector, and retroviral supernatants were produced by transiently cotransfecting 293 GP cells with the retroviral vector and a plasmid encoding the VSV envelope using Lipofectamine Plus (Invitrogen Life Technologies) as described.[28] Twenty-four hours later, retroviral supernatants were harvested and used to transduce COS and COS-A2 lines as described.[28] Transduced cells were selected in 2 μg/mL G-418. The untransduced and CAP1 minigene transduced COS cells were maintained in RPMI 1640 complete medium.

Generation of Lymphocyte Microcultures

Leukaphereses were obtained from human donors, and peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation with Lymphocyte Separation Medium (Mediatech). PBMC were suspended at 2×10^6 cells per mL in AIM V human complete medium supplemented with 5 μg/mL KLH (Sigma Aldrich, St Louis, MO) and 25 ng/mL rh IL-7 (Biosource International, Camarillo, CA). CAP1 peptide (YLSGANLNL) or CAP1-6D (YLSGADLNL) peptide (Synthetic Biomolecules, San Diego, CA) was added to a final concentration of 10 μg/ml. Peptides used were > 95% pure as determined by mass spectrometry. On culture day 3, rh IL-2 (Cetus, Berkley, CA) was added to a final concentration of 30 IU/ml. On culture day 7, aliquots of 5×10^3 cells were suspended in 200 μl of complete medium in 96 well U-bottom microculture plates. Cell aliquots were stimulated in vitro with 1×10^5 irradiated (10 Gy) peptide-loaded autologous PBMC. Microcultures were maintained in complete medium plus 30 IU/mL rh IL-2, and in vitro stimulations were repeated with 1×10^5 irradiated (10 Gy) peptide-loaded autologous PBMC on days 14, 21, 28, and 35. Microcultures were tested at weekly intervals for antigen recognition.

Peptide and Tumor Recognition Assays

Cytokine release assays were performed to assess the recognition of peptide-loaded target cells or tumor cell lines. At weekly intervals, cells from each microculture were harvested, divided into 2 equal aliquots and resuspended in fresh medium. T2 cells were loaded with 10 μg/mL antigenic peptide or control gp100:209-217 peptide for 2 hours at 37°C. Microculture aliquots were

cultured overnight with 5×10^4 peptide-loaded T2 cells in 96 well U-bottom plates at 37°C, and supernatants were assayed by enzyme-linked immunosorbent assay (ELISA) for interferon-γ release. Significant peptide recognition was defined by the presence of > 100 pg/mL interferon-γ in the supernatants and secretion of at least twice the interferon-γ secreted during coculture with control targets. TIL 1520, a T cell line that recognizes the melanoma antigen gp100:209-217 expressed on HLA-A*A0201, was used as a positive control for interferon-γ release in each assay. Microcultures that recognized CEA peptide antigen loaded targets were further expanded by restimulating with irradiated peptide-loaded autologous PBMC.

Microcultures that retained their CAP1 reactivity were expanded in AIM V human complete medium containing 30 ng/mL anti-CD3 antibody and 600 IU/mL IL-2 in the presence of 4×10^4 irradiated allogeneic PBMC as described.[29] Expanded T cell lines were tested for recognition of processed antigen by coculturing 5×10^4 T cells with 2×10^4 COS cells, COS-A2 cells, COS cells expressing CAP1, or COS-A2 cells expressing CAP1 overnight in 96 well U-bottom plates at 37°C. Supernatants were assayed for the amount of interferon-γ released by ELISA. Means and standard deviations from multiple assays are shown.

Avidity Testing of Lymphocyte Cultures

The relative avidities of T cell lines for CAP1 peptide were tested in cytokine release assays. T2 cell targets were loaded with limiting concentrations of CAP1 peptide ranging by log from 0.01 to 10,000 ng/mL for 2 hours. After overnight coculture of T cell lines with 5×10^4 peptide-loaded T2 targets, supernatants were assayed for the amount of interferon-γ release by ELISA. The relative amount of cytokine secreted for each target was compared with the maximum cytokine secretion measured for each microculture. Sigmoidal dose response curves and 50% maximum stimulation values were calculated using Graphpad Prism software version 2.1. Means and standard deviations from multiple assays are shown.

Fine Specificity Testing of Lymphocyte Cultures

The fine specificities of T cell lines were assessed using alanine-substituted CAP1 peptide stimulation, as described.[30,31] Eight CAP1 peptide analogues were synthesized (Synthetic Biomolecules) with single amino acid substitutions using alanine at positions 1-4 and 6-9, for example,. *A*LSGANLNL, Y*A*SGANLNL, YL*A*-GANLNL, etc. T2 cell targets were loaded for 2 hours with 10 μg/mL of CAP1 peptide or CAP1 peptide analogues and T cell lines were cultured overnight with loaded T2 targets or T2 targets without peptide loading. Supernatants were assessed for the amount of interferon-γ release by ELISA and the relative amount of cytokine secreted for each target was compared with the cytokine secretion for targets pulsed with CAP1 peptide. Means and standard deviations from multiple assays are shown.

RESULTS

Identification of CAP1 and CAP1-6D–specific T Cells From Donor Lymphocytes

PBMC from HLA-A*A0201-expressing blood donors were stimulated in vitro with the HLA-A*A0201 restricted immunodominant CEA peptide CAP1, or with the heteroclitic peptide CAP1-6D. After a single peptide stimulation of bulk PBMC, each T cell culture was divided into 96 microcultures, and multiple in vitro stimulations were performed using peptide-loaded autologous PBMC. CEA peptide reactivity of the microcultures was tested weekly by interferon-γ release assays (Table 1). CAP1 or CAP1-6D reactive T cell microcultures were established from 4 of the 6 blood donors. The number of stimulations required for generation of peptide reactive cultures and the frequency of peptide reactive microcultures after in vitro peptide stimulation varied among the donor/peptide combinations. Three donor/peptide combinations (donor B/CAP1, donor F/CAP1, and donor F/CAP1-6D) resulted in a high frequency (> 10%) of microcultures that recognized peptide. Characteristic of the microcultures derived from these 3 donor/peptide combinations was the presence of most or all peptide reactive cultures after only 4 stimulations. Five other donor/peptide combinations (donor B/CAP1-6D, donor C/CAP1, donor C/CAP1-6D, donor D/CAP1, and donor D/CAP1-6D) resulted in 1% to 8% peptide reactive microcultures. No reactive microcultures were established from 2 of the donors

TABLE 1. The Relative Prevalence of CAP1 and CAP1-6D Reactive T Cells in Normal Blood Donors

	Reactive Cultures (n/96 wells, %)					
	CAP1			CAP1-6D		
	d.26	d.33	d.40	d.26	d.33	d.40
Donor B	17	17	17 (18%)	5	5	5 (5%)
Donor C	0	1	2 (2%)	5	7	7 (7%)
Donor D	0	0	8 (8%)	0	1	1 (1%)
Donor F	11	11	11 (11%)	6	13	14 (15%)
Donor K	0	0	0 (0%)	0	0	0 (0%)
Donor Z	0	0	0 (0%)	0	0	0 (0%)
Total	28	29	38 (7%)	16	26	27 (5%)

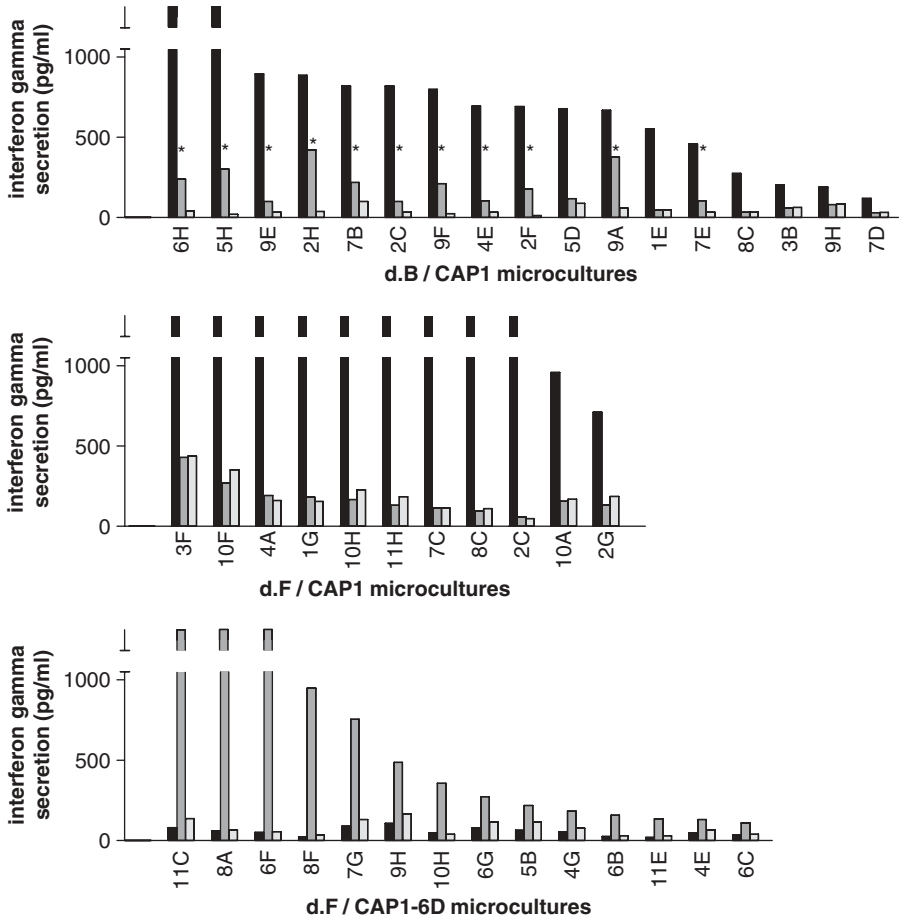

FIGURE 1. Microcultures from Donor B/CAP1 cultures, Donor F/CAP1 cultures, and Donor F/CAP1-6D cultures that maintained reactivity against the stimulating antigen for at least 2 consecutive weeks were tested for reactivity against the heteroclitic peptide antigen. T2 cell targets were loaded with 10 μg/mL of CAP1 or CAP1-6D peptide for 2 hours. Cytokine release assays were performed on supernatants after overnight coculture of microcultures with loaded T2 targets. Reactive cultures responded to target stimulation with cytokine release greater than twice that released in response to irrelevant gp100:209 antigen. Black bars show reactivity against CAP1 loaded targets, dark gray bars against CAP1-6D loaded targets, and light gray bars against gp100:209 loaded targets. Asterisks indicate cross-reactive cultures. Cocultures with >1000 pg/mL cytokine are indicated with broken bars.

(donor K and donor Z) using either CAP1 or CAP1-6D peptide in multiple attempts. In sum, neither CAP1 nor CAP1-6D was a consistently superior immunogen, and the ability to generate peptide reactive cultures was both peptide and donor specific.

Lymphocyte Cross-reactivity Against Heteroclitic Peptide Antigen

Previous studies have shown that selected CAP1-specific T cells efficiently cross-recognize the heteroclitic peptide antigen CAP1-6D.[18] The ability of peptide reactive microcultures to recognize the heteroclitic peptide antigen pair CAP1 and CAP1-6D was tested for the 3 donor/peptide combinations with a high frequency of reactive cultures. Microcultures that recognized CAP1 or CAP1-6D in cytokine release assays were stimulated in vitro an additional time and tested for recognition of targets expressing the stimulating antigen, the heteroclitic peptide analog, or control peptide antigen (Fig. 1). Among 17 microcultures generated by simulation of donor B cells with CAP1 peptide, 11 also recognized CAP1-6D in interferon-γ release assays (supernatants with > 100 pg/mL interferon-γ and levels at least twice that released with control peptide loaded targets). For each cross-reactive microculture, the stimulating peptide resulted in more interferon-γ release than the heteroclitic peptide. The 6 microcultures that did not show cross-reactivity were among the least reactive against the stimulating peptide, suggesting that the assay was less sensitive for cross-reactivity at low cytokine secretion levels.

Among 11 microcultures generated by simulation of donor F cells with CAP1 peptide and 14 microcultures generated by simulation of donor F cells with CAP1-6D peptide, no cultures showed cross-reactivity against the heteroclitic antigen. Thus, the cross-reactivity of peptide-specific microcultures tested here was a property of the donor/peptide combination, and cross-reactivity was not universal. CAP1-reactive T cells from donor F were poorly stimulated by the heteroclitic antigen CAP1-6D. Conversely, CAP1-6D reactive T cells from this donor were not stimulated by the native peptide antigen CAP1.

Lymphocyte Avidity Against CAP1 Peptide Loaded Targets

To characterize the avidity of CAP1-reactive cells generated from blood donors, the CAP1-specific microcultures described above and additional microcultures using the same donor/peptide combinations were expanded via anti-CD3 antibody stimulation. Six resultant T cell lines from donors B and F that maintained antigen

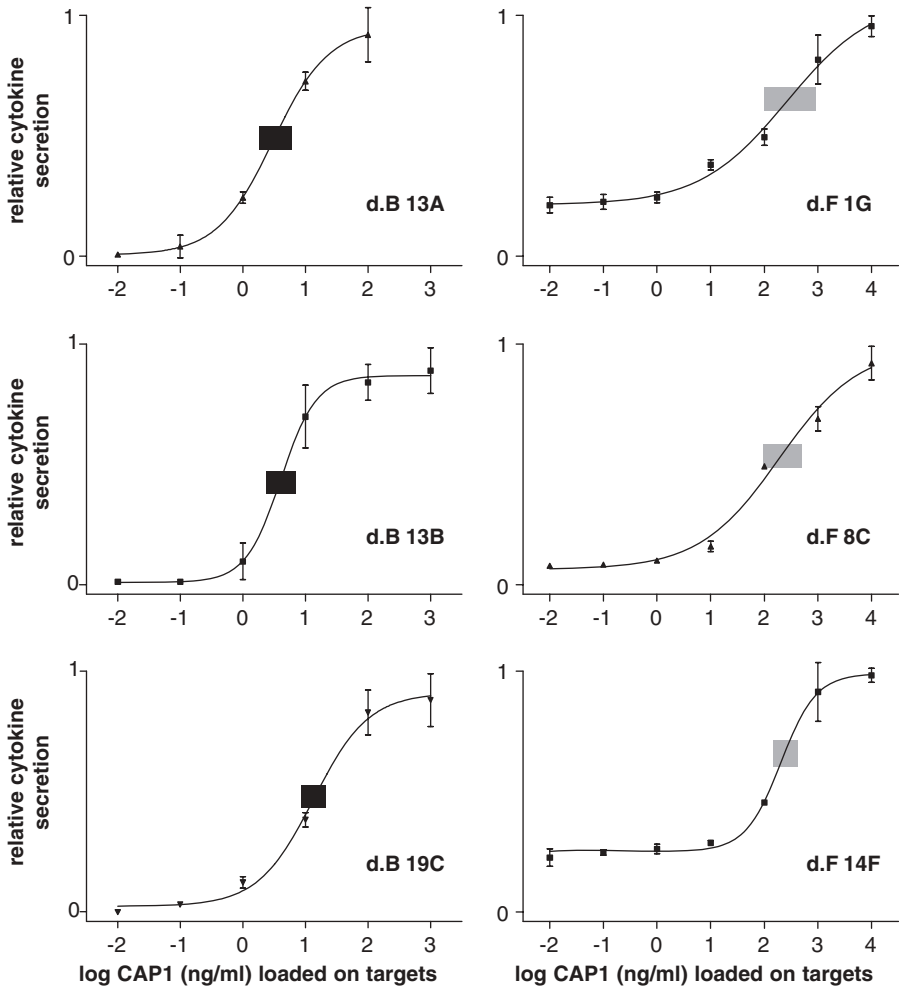

FIGURE 2. Microcultures from donor B and donor F were restimulated 4 additional times (total 8 to 10 stimulations), then rapidly expanded in complete medium using anti-CD3 antibody (30 ng/ml), IL-2 (600 IU/ml), and irradiated allogeneic PBMC at 500:1. T2 cell targets were loaded with limiting concentrations of peptide ranging by log from 10 pg to 10 μg/mL for 2 hours. Cytokine release assays were performed on supernatants after overnight coculture of expanded T cell lines with loaded T2 targets. Results are expressed relative to the maximum cytokine secretion measured for each line. Higher CAP1 peptide concentrations than shown did not increase target recognition. Results are representative of multiple assays. The 95% confidence intervals for half maximum stimulation are shown in black (donor B) or gray (donor F).

reactivity were tested in interferon-γ release assays using targets loaded with decreasing concentrations of peptide antigen. The avidity of microcultures was standardized relative to the maximal cytokine secretion for each culture (Fig. 2). T cell line 13A from donor B was maximally stimulated by targets loaded with 100 ng/mL CAP1 peptide. Half maximum interferon-γ release was seen with T2 cells loaded with 2 to 6 ng/mL of peptide. Two other T cell lines from donor B (13B and 19C) had similar avidity profiles. Half maximum interferon-γ release was observed from these lines after stimulation by T2 cells loaded with 2 to 7 or 8 to 21 ng/mL peptide, respectively. In contrast, T cell lines from donor F (1G, 8C, and 14F) had considerably lower avidity: each of these T cell culture required 10,000 ng/mL peptide loading of targets for maximal stimulation and half maximum interferon-γ release was observed when stimulated by T2 cells loaded with 90 to 800 ng/mL of peptide. In summary, the relative avidities of several reactive microcultures from an individual donor were similar. None of the microcultures were of high avidity, commonly described as the ability to recognize T2 cells loaded with nanomolar (1.1 ng/mL CAP1) concentrations of peptide.

Lymphocyte Fine Specificity Against Native CAP1 and Substituted CAP1 Peptides

The fine specificities of the CAP1 peptide reactive T cell lines from donors B and F were examined by testing each line for the ability to recognize a panel of alanine-substituted CAP1 peptides. The recognition of substituted peptides by each microculture is shown relative to the amount of interferon-γ secreted in response to stimulation with the native CAP1 peptide, which has an alanine at position 5 (Fig. 3). Three T cell lines from donor B showed similar fine specificity patterns. Substitutions at peptide position 2 or 3 resulted in weak peptide recognition whereas substitutions at position 7 or 9 resulted in strong peptide recognition. Substitutions at position 4, 6, or 8 were nonpermissive, resulting in no recognition of the peptide by any of the donor B microcultures. Three T cell lines from donor F shared a fine specificity pattern that was distinct from the pattern exhibited by donor B microcultures. Substitutions at peptide position 2 or 6 resulted in weak peptide recognition of targets, and substitutions at position 9 resulted in strong peptide recognition. Alanine substitutions at other positions were nonpermissive. The fine

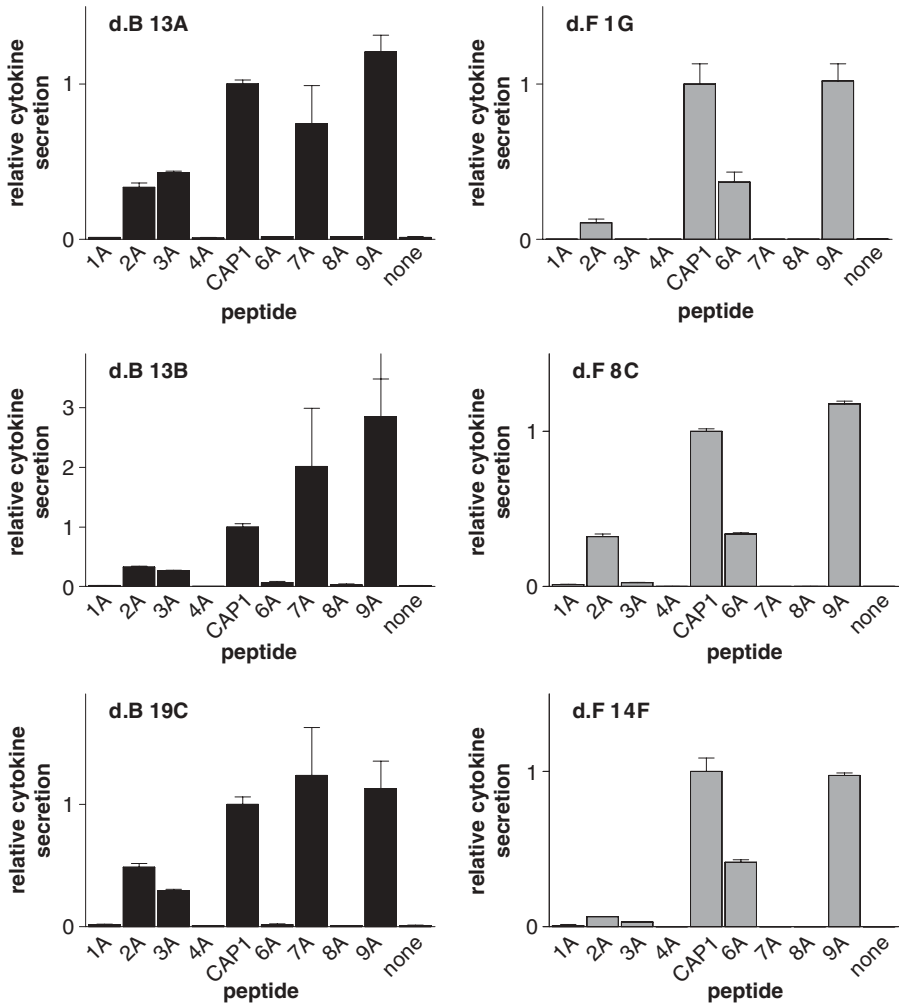

FIGURE 3. The fine specificities of expanded T cell lines from donor B (black bars) and donor F (gray bars) were assessed using alanine substituted CAP1 peptide stimulation. T2 cell targets were loaded for 2 hours with 10 μg/mL of CAP1 peptide or CAP1 peptide altered by alanine substitution at a single amino acid position. Cytokine release assays were performed on supernatants after overnight coculture of lines with loaded T2 targets or T2 targets without peptide loading. The results for each line are expressed relative to the cytokine secretion measured after coculture with CAP1 loaded targets. Results are representative of multiple assays.

specificity results demonstrated that the repertoires of readily expanded CAP1-specific cells from donor B and F were narrow and were donor-specific.

Lymphocyte Reactivity Against CAP1 Expressing Tumors

To confirm that T cell lines from donor B and F could recognize antigenic peptide presented through the class I pathway, cytokine release assays were performed using tumor targets transduced with a construct encoding the CAP1 peptide (Fig. 4). All 3 T cell lines from donor B recognized endogenously produced CAP1 peptide in an HLA-A*A0201-restricted manner. Only 1 of 3 donor F T cell lines showed weak recognition of the CAP1 transduced HLA-A*A0201$^+$ COS targets.

DISCUSSION

Most CEA-directed immunotherapy strategies require a population of effector T cells capable of expansion and tumor cell killing in vivo. Here, we have used in vitro studies of T cell antigen recognition and function to characterize the CAP1-reactive T cell population in HLA-A*A0201-expressing individuals. The goals of this study were to evaluate the variability of the T cell response to CEA among individuals and to characterize the identified responses in detail. Considering that the altered peptide ligand CAP1-6D is currently in use in clinical trials, an important component was the analysis of responses to the CAP1-6D and the native CAP1 peptide. A consistent ability of CAP1-6D to generate cross-reactive T cell responses would have supported the use of CAP1-6D over CAP1 in all future trials. In addition to cross-reactivity, the avidity of CEA reactive cells and the breadth of the repertoire were analyzed to estimate the potential for generating a robust antigen response with CEA-directed immunotherapy. After a detailed analysis of only 6 blood donors, we were able to see that the T cell responses to CAP1 and CAP1-6D were highly variable among individuals.

A readily expandable CAP1-reactive population was identified in 4 of 6 individuals, and the response was vigorous in 2. The heteroclitic antigen CAP1-6D was not substantially superior to the native peptide CAP1 in expanding specific T cells with the techniques used here. In fact, cross-reactivity of CAP1 or CAP1-6D reactive cells for the heteroclitic epitope was not seen in 2 of the 3

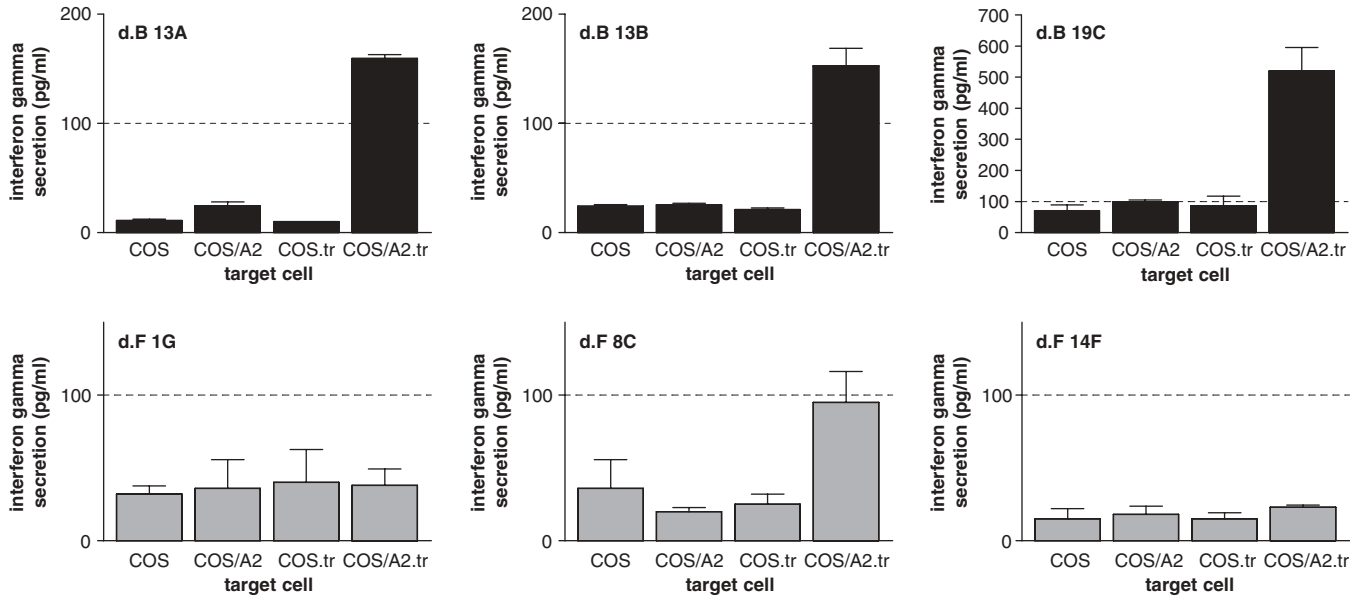

FIGURE 4. The ability of expanded T cell lines from donor B (black bars) and donor F (gray bars) to recognize naturally processed and presented antigen were assessed using transduced mammalian tumor targets. COS cells were stably transduced with the human HLA-A2 gene and/or with a vector containing the DNA sequence for the CAP1 peptide epitope preceded by the Kozak consensus sequence and an ATG start codon. Cytokine release assays were performed on supernatants after overnight coculture of lines with tumor targets (COS), targets expressing HLA-A2 (COS/A2), targets expressing the CAP1 epitope (COS.tr) or targets expressing both HLA-A2 and the CAP1 epitope (COS/A2.tr). Results are representative of multiple assays.

readily expandable populations, demonstrating that CAP1-6D stimulation may selectively expand cells incapable of recognizing native antigen. Individual T cell lines from expandable populations recognized substituted antigenic peptides with a similar pattern, suggesting narrow repertoires for CAP1-reactive T cells in these individuals. Antigen-specific cell lines showed modest functional avidity and weakly recognized CAP1-expressing tumors in assays of cytokine secretion. Taken together, these studies suggest that only a subset of individuals has the potential to benefit from CEA-directed heteroclitic antigen immunotherapy.

T Cell Cross-reactivity After Heteroclitic Antigen Stimulation

Heteroclitic peptide antigens have been reported for tumor-associated antigens from several human cancers.[4–8,10,16,18] Immunotherapy with altered peptide ligands has the potential to stimulate cells that are not stimulated by the native antigen in vivo. Once primed, these T cells may be able to recognize native antigen on target cells. It is reasonable to hypothesize that the frequency of cells recognizing altered peptides is higher than that of cells recognizing native antigen due to positive thymic selection during development, or through overcoming suppression by recurrent stimulation with antigen in vivo. Experimentally, some altered peptide ligands have been shown to be superior binders to MHC class I molecules, better stimulators of effector cell function, or capable of eliciting superior responses against native antigen in human studies and animal models.[5–7,11,13–17] Our results confirm the findings of others: that T cell populations capable of recognizing self antigen can be identified in most individuals. Further, they suggest that the native antigen may be a superior immunogen for effecting a tumor response in some individuals. We found that the altered peptide CAP1-6D was not more likely to be recognized than CAP1 in vitro among blood donors. Furthermore, T cells from donor B that were stimulated by altered peptide could not recognize native antigen, and altered peptide failed to stimulate T cells in the same donor that recognized CAP1. The ability for a T cell to exhibit cross-reactivity may be influenced by T cell avidity. We found that among donor B microcultures, only cells secreting high amounts of interferon γ (> 400 pg/ml) exhibited cross-reactivity. Several strategies, such as altering the tumor microenvironment, abrogating suppression, and improving T cell costimulation, have been used to increase T cell recognition of CEA in recent human and animal studies.[32,33] The success of these strategies may be due in part to recruitment of additional cross-reactive T cells by enhancing their avidity in vivo.

The Contribution of TCR Structure to T Cell Cross-reactivity

The main determinant of T cell cross-reactivity is the TCR structure. We found that the fine specificity of CAP1-reactive microcultures from a single donor were similar, suggesting that the TCR structure of reactive cells

in the cultures were similar. The most likely explanation for these findings is rapid expansion of single T cell clonotypes in vitro, resulting in the presence of sister clones in several microcultures from each donor. Alternatively, the antigen experience of the donor may result in an oligoclonal repertoire with similar fine specificity patterns. If, as proposed by other investigators, the pool of cross-reactive cells in an individual's repertoire is influenced by exposure to environmental antigen mimics,[4] structural similarities among cross-reactive cells may be required for recognition of both the mimic and the native antigen.

When used for immunotherapy, heteroclitic peptides acting as antigen mimics likely result in a skewing of the repertoire. Antigen mimics may skew the T cell repertoire away from native antigen reactivity, as demonstrated here by the cultures generated in vitro from donor B. Importantly, alterations that do not change the conformation of the MHC-peptide at its interface with the TCR, such as those at MHC anchor residues, are unlikely to result in narrowing of the repertoire or skewing of the repertoire away from native antigen reactivity.[3] Peptide epitopes that have been altered to improve MHC binding may therefore be preferable for therapy to those that affect the peptide-TCR interface.

Implications of a Limited T Cell Repertoire Against CAP1

The frequency of CEA-specific T cells in blood donors and cancer patients is low, and expanding these cells for analysis or cell therapy has proven difficult.[34] We found that a microculture technique for in vitro stimulation was necessary to reproducibly obtain reactive populations from our blood donors. By limiting the number of cells in each microculture after the first in vitro stimulation, the starting precursor frequency was artificially raised to 1/5000 for all potentially reactive cultures. Nevertheless, we were still unable to expand CAP1-reactive cells from 2 of our 6 donors. By comparison, the expansion of MART-1/Melan A reactive populations by in vitro stimulation of cells from blood donors has not required the microculture technique (data not shown). In our donors, not only was the frequency of CAP1-reactive cells low, but the diversity of the repertoire was narrow when the fine specificity of individual cultures was assessed, and the avidity of our 6 lines for CAP1 was moderate or low. Relative difficulties generating and maintaining antigen-reactive T cell populations in vitro are likely contributors to the slow progress in immunotherapy for tumors other than melanoma.

CEA Precursors and CEA-directed Therapy in Cancer Patients

Whether the variation in CEA-specific T cell frequencies reported here is representative of that in cancer patients is unclear. The functional T cell repertoire could be significantly altered in patients with cancer. Under certain conditions, tumors may represent a site of autoimmunization through antigen expression in an immunogenic environment, raising precursor frequencies.[35] Alternatively, the tumor-bearing state may itself lead to suppression of T cell responses, as has been demonstrated in several animal models.[36–38] Further, the specific effect of cancer on an immune response may be influenced by the tumor burden, that is, the presence of microscopic disease versus clinically apparent metastatic disease.

Recent clinical trial evidence shows that CEA-directed heteroclitic peptide therapy can be effective in some individuals. Fong et al[17] reported 1 complete radiologic response, 1 complete serologic response, and 1 mixed radiologic response among 12 patients with metastatic cancer treated using CAP1-6D loaded autologous dendritic cell vaccines. Regressions of tumors after CEA-directed therapy suggest that immunotherapy for human adenocarcinomas should be pursued, with efforts made to incorporate novel strategies already in use for the treatment of melanoma. This study suggests that patients with T cells capable of a robust response to CEA-directed therapy could be selected for treatment using in vitro techniques. If validated in future studies, a correlation between a priori T cell precursors and immune responses to therapy may provide a useful screening criteria for entry into trials. For patients with poor CEA responses at screening, the use of nonmyeloablative chemotherapy as an adjuvant to immunotherapy or adoptive therapy with gene-modified effector cells may provide additional benefit.

Any conclusions from this study regarding the CAP1-specific T cell response must be considered in the context of its limitations. In vitro stimulation methods are subject to significant artifact, and extrapolating these findings to an in vivo response after active immunotherapy may be an oversimplification. Further, the cytokine release assays performed in this analysis are not as sensitive or quantitative as single-cell methods of assessing antigen recognition, such as tetramer binding or immunofluorescence staining of intracellular cytokine. Therefore, low-frequency cells that recognized antigen ex vivo but were not readily expanded in culture were not reflected in this analysis. We believe that cells that recognize antigen and are also able to be expanded are of primary importance for an immune response. Conversely, tetramer binding cells that do not produce cytokine or expand with antigen stimulation are less important, because they are less susceptible to manipulation by immunotherapy. Finally, it can be noted that the T cell lines identified here only weakly recognized processed antigen on tumors.

In summary, we found that the T cell response to in vitro stimulation with the HLA-A*A0201-restricted CAP1 peptide from CEA, or by the heteroclitic peptide CAP1-6D, varied widely among individuals. The dominant expanding T cell populations in these individuals did not always recognize heteroclitic antigen, and multiple T cell lines from individuals were from a narrow repertoire. Among the individuals studied, reactive T cell

populations were of low to intermediate avidity. The expandable T cell repertoire may be a central contributor to the limited responses seen with CEA-directed immunotherapy to date. Additional treatment strategies designed to alter or expand the T cell repertoire against CEA expressing tumors should be considered for trials.

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