Molecular Mimicry of Carcinoembryonic Antigen by Peptides Derived from the Structure of an Anti-Idiotype Antibody

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ABSTRACT

Our goal was to use carcinoembryonic antigen (CEA) as a target for immunotherapy in CEA-positive cancer patients who are all immune tolerant to the native antigen. We isolated and characterized an anti-idiotype monoclonal antibody 3H1, which mimics a distinct and specific epitope of the M, 180,000 CEA and can be used as a surrogate for CEA. In Phase Ib clinical trials in a group of 23 advanced colorectal cancer patients, 3H1 induced both humoral and cellular anti-3H1 responses, as well as anti-CEA immunity. To study the cellular immunity invoked by 3H1 at the molecular level, we have cloned and sequenced the cDNAs encoding the variable heavy and light chains of 3H1 and deduced the amino acid sequences of the encoded proteins. To identify any cross-reactive peptides of 3H1 and CEA, we compared the amino acid sequences of 3H1 with those of CEA and found several regions of homology in 3H1 heavy and light chain variable domains, as well as in the framework regions. To search for potential cross-reactive T-cell epitopes, a number of peptides were synthesized based on 3H1/CEA homology and were used as stimulants in cell proliferation assays, using peripheral blood mononuclear cells from a group of 3H1-immunized CEA-positive cancer patients in the adjuvant setting. Two partially homologous peptides, designated LCD-2 (from 3H1) and CEA-B (from CEA), were identified in 10 of 21 adjuvant patients by strong proliferation responses (stimulation index, 3–50-fold), which were extensively studied in five of these individuals over an extended period of time (12–24 months). We saw no correlation with the MHC class I haplotype of the patients. Analysis of the subtype of the responding T cells demonstrated that primarily CD4+ T cells were stimulated by both 3H1 and 3H1-derived peptides. Interleukin 2, interleukin 4, and IFN-γ were assayed in the culture medium of peripheral blood mononuclear cells stimulated with 3H1, CEA, and LCD-2 to determine the T-cell helper subset induced by these stimulants. The in vitro responses were mainly associated with secretion of IFN-γ, which suggested that the induced T cells were most likely Th1 type. Future studies will include the design of second-generation LCD-2 and CEA peptides to further enhance antigenicity, to characterize the responding T-cell populations more fully, and to test refined peptides for immunogenicity.

INTRODUCTION

A potential target for immunotherapy, which may be useful for the treatment of several carcinomas, is CEA. Cancer patients are tolerant to CEA, and triggering an active immune response to such antigens represents a major challenge in cancer therapy. Several laboratories are involved in the design of CEA-based vaccines for cancer patients (1–4). A recombinant vaccinia virus expressing CEA has been used as a tumor vaccine (2). This study demonstrated that CEA can be processed endogenously by human tumor cells. A specific CEA peptide (CAP-1), which is processed by the tumor cells, can be presented by the MHC class I molecule, HLA-A2, to generate cytotoxic T cells that are specific for CEA-positive colorectal cancer cells. In another study, plasmid cDNA encoding CEA was used as a vaccine for the therapy of colorectal cancer patients (3). We have used an anti-idiotype antibody vaccine that acts as a surrogate for CEA to activate specific immunity in CEA-positive cancer patients (1, 4). This idea is based on Jerne’s network hypothesis (5), which proposes that, following immunization with an antigen, several types of anti-idiotypic antibodies are elicited. A subset of these antibodies, designated Ab2β, acts as the internal image of the original antigen and mimics its molecular features. Ab2β have been used as surrogate antigen for immunizations against specific bacterial, viral, and parasitic infections, as well as cancer (reviewed in Ref. 6).

The anti-idiotype antibody we have generated is designated 3H1, which mimics a distinct and specific epitope of CEA (7). In Phase Ib clinical trial, hyperimmune sera from 17 of 23 advanced patients vaccinated with 3H1 demonstrated an anti-idiotypic Ab3 response, and 13 of these responses were demonstrated to be anti-CEA (Ab1’). The antibody response was polyclonal, and 11 mediated antibody-dependent cellular cytotoxicity. Ten patients had idiotypic T-cell responses, and 5 of these 10 also had specific T-cell responses to CEA (4). We believe that the T-cell response observed against CEA was based on the recognition of processed 3H1 linear peptides that have homology to the CEA sequence. In a murine model, we demonstrated that immunization of naive mice with 3H1 vaccine-induced humoral and cellular anti-3H1, as well as anti-CEA immunity (8). Mice immunized with 3H1 were protected against challenge with lethal doses of CEA-positive tumor cells, whereas no protection was observed when 3H1-vaccinated mice were challenged with CEA-negative tumor cells or when mice were vaccinated with an unrelated anti-idiotype antibody and challenged with CEA-positive tumor cells (8).

To study the molecular basis of the emergence of anti-CEA T-cell responses in the animal model, as well as in patients vaccinated with 3H1, and to develop peptide-based second-generation cancer vaccines, we sought to identify cross-reactive peptides of CEA and 3H1. We have reported in this communication: (a) the sequence of 3H1, (b) computer-aided design of eight candidate cross-reactive and control peptides based on 3H1/CEA homology, and (c) evaluation of proliferative T-cell responses to these peptides in a group of 3H1-immunized patients at the adjuvant setting. We observed that two peptides stimulated proliferation of PBMCs from a number of patients at multiple times during the course of 3H1 therapy but had no effect on pretherapy PBMCs.

MATERIALS AND METHODS

Materials. Tag DNA polymerase was obtained from Promega (Madison, WI). Mixed oligonucleotides for PCR amplification of immunoglobulin variable domains, NovaBlue competent cells, and plasmid pT7Blue(R) were purchased from Novagen (Madison, WI). Sequense Version 2.0 sequencing kit was from United States Biochemical (Cleveland, OH). SuperScript Preamplification kit was pur-
chased from Life Technologies, Inc. (Gaithersburg, MD). Oligonucleotides used for DNA sequencing were synthesized by the Macromolecular Structural Facility of the University of Kentucky at Lexington.

**Determination of the Nucleotide Sequences of Variable Heavy and Light Chains of 3HI.** Total RNA was isolated from 1 x 10^7 3HI-producing hybridoma cells (9). First strand cDNA was synthesized using SuperScript Premplification kit. DNA fragments encoding the V_H of 3HI were then amplified by the PCR using the mixed oligonucleotide forward primer 5'-GGGAATTCATGRAATGSASCTGGGTYWTYCTCTT and the reverse primer 5'-CCCAAGCTTCCGAGGCCKGARKTARACIGRTGG, corresponding to sequences of the leader (signal peptide) region amino acids 20 to 13 and the y constant region amino acids 126-119 of IgG. The cDNA fragment encoding the 3HI V_H was similarly amplified. For the light chain cDNA, the mixed oligonucleotide forward primer used was 5'-ACTAGTC-GACATGTTCCWCSACTCAGTCTCCTG, and the reverse primer was 5'-CCCAAGCTTACTGATGTGGAGATGG, corresponding to amino acids 20 to 13 amino acids of the light chain leader sequence and amino acids 126-119 of the mouse K constant region (I = inosine, R = A or G, Y = C or T, K = G or T, S = C or G, and W = A or T), respectively.

The fragments of cDNA amplified were subcloned into pT7Blue(R) plasmid and NovaBlue competent cells were transformed using a protocol provided by the supplier (Novagen). Plasmid DNA from selected clones was prepared by the miniprep procedure (10), and the DNA sequence of the double-stranded plasmid was determined by Sequenase Version 2.0 kit. The sequence of the DNA insert in the plasmid was determined from both orientations using T7 promoter primer (5'-TAAATAGCTACTAATAGGG) and U-19 primer (5'-GTTTTCCCAGTCACGACGT). To determine the entire sequence of the amplified cDNA fragments, oligonucleotides were synthesized corresponding to the sequences of 5' and 3' ends of the sequence obtained in the first round and used for a second round of sequencing. At least 10 clones were chosen for sequence determination. The sequence shown by 70-80% of the clones was accepted as the sequence of the cDNA. Sequences obtained were analyzed for open reading frame using IG suite (IntelliGenetics, Inc., Mountain View, CA). A homology search was carried out in a nonredundant protein and Kabat database (11) in the National Center for Biotechnology Information databank using the Blastp program (12).

**Purification of Anti-Idiotype mAb 3HI.** 3HI (IgG1, κ) was purified from the ascites by affinity chromatography on protein A-CL Sepharose 4B column (Pharmacia Biotech, Piscataway, NJ) and DEAE ion-exchange chromatography. The purity of the isolated immunoglobulin (>95%) was determined by SDS-PAGE and high-pressure liquid chromatography.

**Verification of the cDNA Clone by Amino Acid Sequence.** For the amino acid sequence determination of 3HI variable regions, 50 μg of purified 3HI was diluted with sample loading buffer [50 mm Tris-HCl (pH 6.8), 1% SDS, 1% glycerol, and 0.1% β-mercaptoethanol] and heated to 100°C for 3 min. The denatured protein was loaded onto a 7.5% polyacrylamide gel (Bio-Rad Mini Protean II Dual Slab Cell) containing SDS and subjected to electrophoresis at 200 V for 1 h (10). Proteins in the gels were transferred to polyvinylidene difluoride membranes (13) at 150 mA overnight. The transfer buffer contained 25 mm Tris, 192 mm glycine, and 20% (v/v) methanol. The membranes were stained by quick dipping in 0.5% Coomassie Brilliant Blue in 50% methanol-5% acetic acid, followed by rinsing in a solution containing 40% methanol plus 10% acetic acid. After drying the membranes at room temperature, the stained heavy and light chain bands were excised with a clean razor blade. Proteins on the membrane slices were subjected to NH2-terminal amino acid sequence determination of 3HI variable regions. 50 μg of purified 3HI was digested with 0.1 μg of trypsin in 0.1 μg of trypsin in 0.1 M citrate buffer, pH 4.0, containing 10% acetic acid for 30 min at room temperature. The digested peptides were separated by HPLC.
RESULTS

Amino Acid Sequence of 3H1 Variable Heavy and Light Chains. Although a majority of the analyzed clones had the same DNA sequence, suggesting that these clones were derived from mRNA present in the 3H1 hybridoma, we confirmed the origin of these clones. 3H1 heavy and light chains were purified and the sequences of 15 NH2-terminal amino acids were determined from these proteins. These amino acid sequences were exactly what would be predicted from the cDNA sequence of the mature chains. To examine this possibility, the complete amino acid sequence of 3H1 to have linear homology to mouse IgG Eo'CL (Ref. 11, on-line version).

Amino acid sequences of the heavy (A) and the light (ß) chains were deduced from the cDNA sequence, as described in "Materials and Methods." CDR and framework domains were assigned according to Ref. 11. Underlined sequences, sequences obtained from the hybridoma, were confirmed the origin of these clones. 3H1 heavy and light sequences were determined from these proteins. These amino acid sequences were obtained from the NH2-terminus of the purified heavy and light chains of 3H1 for clone verification.

Sequence of 3H1 Heavy Chain

MEWSWVILFLSGLGTAVHS (Leader)
EYOLQGSGEPVLGKASLKLICEASBGYSLT (Framework #1)
AYTMN (CDR #1)
WKQSGHKSLEWGG (Framework #2)
LINPSGDNTYSSQKFTG (CDR #2)
KATLTVDRSSATAMYELLSLSTEDSAYYCVI (Framework #3)
TPPVYFYFDV (CDR #3)
WGAGTVTYVSS (Framework #4)
AKTTPPSVY (Constant region)

Sequence of 3H1 Light Chain

MVSTAOFLGILLWFPGIKS (Leader)
DIKMTQGSPSSMYASLGTERVITTC (Framework #1)
KASQDINGYLN (CDR #1)
WFQQEPGKSPKTL (Framework #2)
RANKLD (CDR #2)
GYPERSFGSGSQQVYSLTISSEYDGMGTYWC (Framework #3)
LQFDEPWPW (CDR #3)
FGGKTQKLEIK (Framework #4)
RADAAPTIPSQPS (Constant region)

Fig. 1. Amino acid sequences of the variable domains of 3H1 heavy and light chains. Amino acid sequences of the heavy (A) and the light (B) chains were deduced from the cDNA sequence, as described in "Materials and Methods." CDR and framework domains were assigned according to Ref. 11. Underlined sequences, sequences obtained from the NH2-terminal ends of the purified heavy and light chains of 3H1 for clone verification.

CEA (15) was retrieved from a protein database [Protein Identification Resource (PIR) accession no. A36319] for comparison with the 3H1 amino acid sequences. Heavy and light chain CDRs and framework regions were individually aligned to the complete open reading frame of CEA using the IG suite. Results of this alignment are shown in Fig. 2.

Comparison of the linear sequence of 3H1 and CEA may also identify the common epitope of these proteins recognized by the Ab1 anti-CEA mAb 8019 (7). Similarity of this epitope could be direct, as shown above, or could be in reverse orientation (16). To analyze the human CEA consists of three homologous repetitive domains (15). Part of these repeat domains are shown in Fig. 2C. One 3H1 peptide in particular (LCD-2) showed high homology to this CEA region. The homology of the 3H1 peptide is maximum with repeat II, showing 4 of 10 identity (Fig. 2C).

Analysis of Hydrophobic Complementarity to Identify Idiotype-Anti-Idiotype Contact Sites. A program has been written to identify interaction sites between proteins (17). This program scans and compares the hydrophobic profiles of interacting proteins and is based on the Kyte and Doolittle scoring of individual residues (18). If the contact sites are surface accessible in the folded proteins, they may constitute the binding sites of the interacting proteins. We used this program to identify the common sequences of 3H1 and CEA recognized by the Ab1 mAb 8019. The sequences of the variable regions of 8019, 3H1, and the open reading frame of CEA were entered into the program for comparison of hydrophobic profiles. A sequence PIGN was identified as one of the idiotype-anti-idiotype contact points by this analysis, which was present in CEA repeat region III shown in Fig. 2C and shows, in reverse orientation, two exact and two partial homologies to the 3H1 light chain region around CDR2. Results of the amino acid sequence alignment and reverse hydrophathy analysis suggested that CDR2 of the 3H1 V\textgreek; and the region of CEA repeat shown this analysis, which was present in CEA repeat region III shown in Fig. 2C may be the common epitope recognized by the anti-CEA Ab1, 8019. We, therefore, designed two peptides for analysis, one from 3H1 (LCD-2) and the other from CEA (CEA-B), containing the common sequence LIDG (Table 3).

Design of Peptides Based on 3H1/CEA Sequences with Potential T-Cell Epitopes. Besides LCD-2 and CEA-B, we designed and synthesized a number of other peptides (Table 3) and set up an assay to test their

Table 2 Characteristics of the CEA-positive cancer patients undergoing 3H1 immunotherapy

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Diagnosis</th>
<th>HLA-A</th>
<th>HLA-B</th>
<th>HLA-C</th>
<th>BW</th>
<th>Time to progression (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM</td>
<td>Female</td>
<td>20</td>
<td>Colorectal</td>
<td>2</td>
<td>18,51</td>
<td></td>
<td>727</td>
<td></td>
</tr>
<tr>
<td>ML</td>
<td>Male</td>
<td>47</td>
<td>Adenocarcinoma of lung</td>
<td>11,26</td>
<td>22,37</td>
<td>4,6</td>
<td>1107</td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>Female</td>
<td>48</td>
<td>Colorectal</td>
<td>2,3</td>
<td>35,44</td>
<td></td>
<td>365</td>
<td></td>
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<tr>
<td>HC</td>
<td>Male</td>
<td>66</td>
<td>Colorectal</td>
<td>1</td>
<td>1</td>
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<td>713</td>
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<td>55</td>
<td>Colorectal</td>
<td>2,28</td>
<td>14,44</td>
<td>4,6</td>
<td>592+</td>
<td></td>
</tr>
</tbody>
</table>

*These five patients at the adjuvant setting were followed serially by T-cell proliferation assay over a long period of time and were treated with 2 mg of aluminum hydroxide-precipitated 3H1, as described in "Materials and Methods."
Fig. 2. Alignment of the amino acid sequences of 3H1 variable domains with CEA. Alignment was performed with the IG suite program using each CDR sequence as the query peptide; the search was then extended manually to framework regions. A, amino acid sequences in direct orientation. B, 3H1 amino acid sequences in reverse orientation. C, alignment with repetitive CEA domains. Amino acid sequences of CEA were retrieved from an existing protein data bank (PIR accession no. A36319) and were numbered according to Oikawa et al. (15). |, exact identity; :, chemical similarity. Bottom, CEA sequences are shown for each alignment with numbering used in Ref. 15. Boldface (C), repetitive domains.

Table 3 Peptides based on 3H1 and CEA sequences

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCD-1</td>
<td>LTAYTMNWV</td>
<td>3H1</td>
</tr>
<tr>
<td>LCD-2</td>
<td>TLIRANRLIDGV</td>
<td>3H1</td>
</tr>
<tr>
<td>HFW-1</td>
<td>GPELVKPGASL</td>
<td>CEA</td>
</tr>
<tr>
<td>CAP-1</td>
<td>YLSGALN</td>
<td>CEA</td>
</tr>
<tr>
<td>CEA-1</td>
<td>PPAQYSLDGN</td>
<td>CEA</td>
</tr>
<tr>
<td>CEA-2</td>
<td>TVTITTVYAEPP</td>
<td>CEA</td>
</tr>
<tr>
<td>CEA-3</td>
<td>GHSRTTVKITT</td>
<td>CEA</td>
</tr>
</tbody>
</table>

Peptides were designed based on the amino acid sequences of 3H1 and CEA, as described in "Materials and Methods." Homologous regions between LCD-2 and CEA-B are underlined.
subsequent experiments, only four peptides LCD-2, CEA-B, CAP-1, and HFW-1 were used. Representative results of PBMC proliferation assays from two patients (CM and VK), before and after four vaccinations with 3H1, are shown in Fig. 3. When the PBMCs were isolated before therapy from patient CM, stimulation was not observed with any of the stimulants, except the positive control stimulant phytohemagglutinin (Fig. 3A). However, following therapy, significant cell proliferation was evident with 3H1, CEA, and an isotype-matched control anti-idiotype antibody, 4DC6, mimicking a T-cell lymphoma epitope (Refs. 21 and 22; P < 0.0001). Stimulation by 4DC6 was significantly lower than 3H1 and CEA (P < 0.0002 versus matched control anti-idiotype antibody, 4DC6, mimicking a T-cell lymphoma epitope (Refs. 21 and 22; P < 0.0001). Stimulation by 3H1, CEA, and 4DC6 was significantly lower than 3H1 therapy and serially at regular intervals during the course of 3H1 therapy.

Analysis of the Subsets of T Cells and Bulk PBMC Populations following Stimulation with Anti-Idiotype Antibodies and Peptides. We analyzed the cell surface phenotype of T cells in the bulk PBMCs of the patients undergoing 3H1 therapy and found that the cells were predominantly CD4+ T cells (data not shown). To study this in more detail, we separated CD4+ and CD8+ T cells from a bulk population of PBMCs obtained from patient GB, who was undergoing immunization with 3H1. The three populations of lymphocytes were expanded in number for more than 30 days using 3H1-pulsed autologous DCs or autologous EBV-transformed B cells as stimulators. At day 46, the lymphocytes were restimulated with autologous DCs pulsed with 3H1, 1A7, LCD-2, or HFW-1. Interestingly, the bulk PBMC and the CD4+ separated lymphocytes responded to DCs pulsed with 3H1 (P < 0.0001), with the CD8+ showing no significant proliferation. Bulk PBMCs and CD4+ lymphocytes also proliferated to the peptide LCD-2 (P < 0.0001), and again, the CD8+ lymphocytes were not responsive. No population of lymphocytes proliferated in the presence of DCs pulsed with the control anti-idiotype antibody 1A7 (P > 0.1) or the control peptide HFW-1 (P > 0.2; Fig. 5A).

Three samples of PBMCs obtained from patient HC, preimmunization (7/94) and two postimmunization samples (9/95 and 1/96) were stimulated in vitro for 30 days with 3H1-pulsed autologous DCs. These PBMCs were then tested for their ability to respond to the anti-idiotype antibodies 3H1, 1A7, and crude lysates made from the CEA-expressing cell lines CY-13 and 624-mel (Fig. 5B). Interestingly, only the postimmunization lymphocytes responded to the immunizing antibody 3H1. No proliferation was observed against 1A7. Significant proliferation was also observed when the PBMCs were stimulated with autologous DCs preincubated with lysates obtained from CEA-expressing cell lines.

Secretion of Cytokines by Stimulated T Cells. Two subsets of CD4+ T helper cells have been identified in the literature which produce distinct sets of cytokines. The Th1 subset secretes IL-2 and IFN-γ, whereas the Th2 subtype secretes IL-4, IL-5, and IL-10. To determine whether the stimulated CD4+ cells constitute predominantly Th1 or Th2 helper cells, the levels of IL-2, IL-4, and IFN-γ were assayed in the culture medium from in vitro stimulated bulk PBMCs isolated from four 3H1-immunized patients. IL-2 and IL-4 were not detected in significant amounts in the PBMC medium from any of these patients by this assay. However, significant levels of IFN-γ was secreted by the PBMCs from these four 3H1-treated patients after stimulation with 3H1 and LCD-2, as well as CEA. Representative data from one patient (CM) are shown in Table 4. These data suggest that CD4+ T cells induced by 3H1 were likely Th1.

<table>
<thead>
<tr>
<th>Schedule</th>
<th>Counts/min</th>
<th>Stimulants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre</td>
<td></td>
<td>3H1</td>
</tr>
<tr>
<td>Post</td>
<td></td>
<td>3H1</td>
</tr>
</tbody>
</table>

Fig. 3. T-cell proliferation of PBMCs from patients with various stimulants. PBMCs were isolated from the patients before (Pre) and after four immunizations (Post) with 3H1 vaccine. Mean cpm incorporated into the cells was determined in the presence of the indicated stimulants, as described in “Materials and Methods.” Peptides were used at 5 μg of dose/well. A, patient CM, representative of group I (Table 1). B, patient VK, representative of group II (Table 1). Sequences of the peptides are described in Table 3.
DISCUSSION

When 3H1 is injected into tumor-bearing hosts, the protein is likely to be internalized and degraded to peptides by host antigen-presenting cells. The degraded peptides bound to the MHC molecules of the host are presented to T cells by the antigen-presenting cells. T cells with appropriate receptors are expanded and expected to constitute the anti-3H1 cytotoxic, helper, and memory cells. For these T cells induced by 3H1 to recognize CEA-positive tumor cells, it is necessary for the amino acid sequence of 3H1 to have linear homology to CEA. We have detected a number of peptides in 3H1 showing linear sequence homology to the nominal antigen CEA. By our methodology, we could only determine linear homology. The number of matches is likely to increase if a loop is introduced into the alignment. A sequence of three amino acids, RGD, in fibrinogen was involved in the binding of fibrinogen to its receptor. A similar sequence, RYD, was identified in the variable heavy chain of the anti-fibrinogen receptor antibody PAC1. Peptides containing RYD inhibited the binding of both fibrinogen and PAC1 to the fibrinogen receptor (23), suggesting that as few as two amino acids may be critical in defining an epitope (24). We, therefore, chose a window of three consecutive amino acid-matches for our alignment experiments. Most of these homologies were in the CDR, with parts extending to the framework regions. CDRs are the sites of idiotope or anti-idiotope docking, and these loop out to form continuous structures exposed on the surface of immunoglobulin to interact with antigen.

The structural basis of antigen mimicry by an anti-idiotype antibody can be conformational, whereas some of them can closely reproduce the antigenic epitope within one of its CDRs. An example of the former type is the mAb E225, an anti-idiotype antibody mimicking an epitope on hen egg lysozyme recognized by the Ab1 mAb D1.3. The crystal structure of E225-D1.3 complex was determined and compared to the binding of D1.3 to E225 with that of D1.3 to hen egg lysozyme (25). E225 did not form an image of hen egg lysozyme epitope at the atomic level. Thus, an anti-idiotype antibody may not need to mimic the antigen at the atomic level to induce antibody that cross-reacts with the antigen.

The second type of antigen mimicry has been demonstrated in several studies. In one study (26), anti-idiotype antibodies were induced against GAT antigen (a random polymer of Glu-Ala-Tyr). These anti-idiotype antibodies contained GAT-like sequences (Glu-Glu-Tyr or Tyr-Tyr-Glu) within the VH CDR3 domains. In the hep-
anti-idiotypic antibodies showed that the sequences of the variable domains of the heavy chains were mostly homologous, but the sequences of the light chains varied considerably. Interestingly, CDR2 of the light chain of 2F10 showed considerable linear homology to a part of the nominal antigen, gp52, between residues 298 and 310. Among the seven amino acid residues of the light chain CDR2, three amino acids were the exact match of this epitope, and two were conservative substitutions.

3H1 can induce anti-CEA immunity in small animals (7, 8) and primates (31), as well as in humans (1, 4). 3H1 appears to be a "true" internal image of the antigen, which functionally and structurally mimics the antigen. If any of these peptides are present as part of a larger peptide with appropriate anchor residues for binding to a MHC molecule, it might have the potential for induction of cytotoxic and/or helper T-lymphocyte proliferation.

NCA, a glycoprotein, is 85% homologous to CEA at the amino acid level and is immunologically cross-reactive to CEA (32). NCA is present on normal lung and spleen. Therefore, any peptide common to CEA and NCA may cause autoimmunity and tolerance. mAb 8019 (Ab1), against which 3H1 (Ab2) was raised, is nonreactive with NCA, suggesting that the epitope recognized by 8019 on CEA is absent on NCA. Scanning of the amino acid sequence of NCA (Ref. 32; GenBank accession no. J03550) demonstrated that the LIDO sequence of our 3H1-derived peptide LCD-2 was not present in NCA. Thus, LCD-2 could be a potential peptide vaccine for immunotherapy of CEA-positive cancer patients.

In an earlier Phase Ib study (1), we reported that 10 of 23 advanced colorectal cancer patients treated with 3H1 vaccine demonstrated anti-idiotype specific T-cell proliferative responses. Of these 10, only five showed T-cell proliferation to CEA. In this adjuvant study, 18 of 21 patients showed anti-idiotype-specific T-cell responses, and 10 of these showed responses to LCD-2, as well as to CEA-B. These patients had low tumor burden when they started 3H1 vaccine therapy and continued on therapy longer than the previous group with high tumor burden, which may explain better immunological responses in these patients. Apparently, there was no correlation with any particular HLA class I haplotype of the patients.

Preliminary tests by flow cytometry analysis of stimulated proliferating PBMC indicated that they are predominantly CD4+ T cells. Specific stimulation of the subsets of T cells in the presence of anti-idiotype antibodies and 3H1-derived peptides confirmed this observation in two patients (Fig. 5). According to the cytokine profile, immune responses have been classified into types I and II (33, 34), respectively, regulated by Thl and Th2 subsets of the CD4+ T helper cells. Among them, Th1 cells produce IL-2, IFN-γ, and tumor necrosis factor-β (35), whereas Th2 cells secrete IL-4, IL-5, IL-6, and IL-10 (36). Secretion of IFN-γ by 3H1 and the peptide-stimulated PBMCs suggested that they were predominantly Th1-type CD4+ cells (Table 4). Secretion of both IL-2 and IFN-γ from PBMCs of colorectal patients treated with an anti-idiotype antibody mimicking the antigen GA733-2 has been reported (37). In another study, administration of a polyclonal anti-idiotype antibody mimicking GA733 in gastrointestinal carcinoma

### Table 4 Cytokines secreted by stimulated PBMCs in the medium

<table>
<thead>
<tr>
<th>Stimulants</th>
<th>IFN-γ (pg/ml)</th>
<th>IL-2 (pg/ml)</th>
<th>IL-4 (pg/ml)</th>
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<tr>
<td>3H1</td>
<td>42.1</td>
<td>3.1</td>
<td>1.2</td>
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<tr>
<td>LCD-2</td>
<td>71.0</td>
<td>4.3</td>
<td>1.0</td>
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<tr>
<td>CEA</td>
<td>28.1</td>
<td>3.6</td>
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<tr>
<td>None</td>
<td>2.3</td>
<td>3.1</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*Cytokines secreted in the medium by stimulated PBMCs of the patient CM were assayed by ELISA, as described in "Materials and Methods."
patients also induced CD4+ T, MHC class II-dependent T cells (38). In our study, PBMCs stimulated with the 3H1 anti-idiotypic antibody or peptides secreted IFN-γ in vitro in a subgroup of patients, suggesting proliferation of Th1 cells.

Traditionally, CD4+ T cells function as helper cells for antibody production. However, these cells have also been shown to have cytolytic functions inducing apoptotic and necrotic cell death (39). Therefore, T cells primed in vivo by 3H1 therapy have the potential for cytolytic activity against CEA-positive tumor cells. Alternatively, 3H1 vaccination may prime Th1-type helper cells, which, in turn, may induce cytotoxic T-cell proliferation by secretion of cytokines, such as IFN-γ.

We recognize that some of the experiments done at the clonal level are preliminary in nature; we could not use a battery of stimulants for lack of adequate numbers of cells. In the future, we plan to more fully characterize the responding T-cell populations and to test refined peptides for immunogenicity. Some of these peptides may serve as second-generation vaccines for the induction of antitumor immunity in a vast majority of cancer patients with CEA-positive tumors.

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Molecular Mimicry of Carcinoembryonic Antigen by Peptides Derived from the Structure of an Anti-Idiotype Antibody

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