

CD4⁺ T Cells from Healthy Subjects and Colon Cancer Patients Recognize a Carcinoembryonic Antigen-specific Immunodominant Epitope

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ABSTRACT

The carcinoembryonic antigen (CEA) is an attractive target for immunotherapeutic purposes because of its expression profile, its role in tumor progression, and its immunogenicity. However, CEA belongs to the CD66 immunoglobulin super-gene family that comprises highly homologous molecules expressed on leukocytes, making CEA a potential autoantigen expressed on hematopoietic cells. We used a MHC class II epitope prediction algorithm (TEPITOPE) to select 11 sequence segments of CEA that could form promiscuous CD4⁺ T-cell epitopes and used synthetic peptides corresponding to the predicted sequences to propagate *in vitro* CD4⁺ T cells from healthy donors and colon cancer patients. CD4⁺ T cells from all subjects strongly recognized the sequence segment (LWWVN-NQSLPVSP), repeated at residues 177–189 and 355–367. Importantly, we demonstrated that this highly immunodominant region contains a naturally processed epitope(s). Cross-recognition experiments with peptide analogues present on the CD66 homologous proteins showed that CEA_{177–189/355–367}-specific CD4⁺ T cells did not recognize the analogues, demonstrating that recognition of the immunodominant epitope is CEA specific. These data suggest that the repertoire of CEA_{177–189/355–367}-specific CD4⁺ T cells might have been shaped by a selective process to exclude CD4⁺ T cells specific for CD66 homologues expressed on leukocyte, while preserving the CEA-specific repertoire. The features of strong immunogenicity and immunodominance in the absence of potential induction of autoimmunity make the identified CEA epitope of particular interest for the development of antitumor vaccines.

INTRODUCTION

CD4⁺ T cells are implicated in fundamental functions in the induction of productive antitumor immunity, comprising: (a) help for CD8⁺ T-cell activation through interaction with APCs,⁶ such as dendritic cells, displaying tumor peptides in association with both MHC class I and II; (b) maintenance of CD8⁺ T-cell memory; (c) indirect effector functions through activation of macrophages and eosinophils and IFN- γ -dependent antiangiogenesis; and (d) direct recognition of MHC class II-positive tumor cells (1–4).

The CEA is a M_r 180,000 glycoprotein expressed at high levels in colon epithelial cells during embryonic development but at significantly lower levels in adult tissue (reviewed in Ref. 5). It is overexpressed in almost all colorectal cancers, 70% of non-small cell lung cancers, and ~50% of breast cancers (5). The CEA, or CD66e,

belongs to the CD66 immunoglobulin super-gene family that comprises also the CD66a, CD66b, CD66c, CD66d, and CD66f molecules (6). Some of them, in addition to epithelial cells, are expressed in normal cells of hematopoietic origin (*i.e.*, leukocytes) and share with CEA regions of high homology (6).

The function of CEA in normal colon epithelial cells and in tumor cells is not entirely clear. It acts as an adhesion molecule with probably different functions in normal *versus* neoplastic colon tissue, because of different localization in the two tissues (7). The altered pattern of localization in tumor cells might help to disrupt intercellular adhesion, with disorganized growth and movement of malignant cells, pointing to a role in the development of the metastatic disease. CEA has been shown also to inhibit cell death (8) and to cooperate in cellular transformation with several proto-oncogenes, such as *BCL2* and *c-myc* (9).

Several reports have shown that CEA is immunogenic. Some groups found evidence of anti-CEA antibodies in colon and breast cancer patients (10–11). The potential to elicit T-cell responses was first suggested by the observation that individuals who had colon cancer often exhibited a delayed-type hypersensitivity response to purified CEA protein (12). More recently, recombinant vaccinia viruses expressing CEA were administered to cancer patients, and CEA-specific T cells were subsequently cloned from these patients, demonstrating that T cells can recognize CEA (13). A few CEA epitopes recognized by CD8⁺ T cells (14–21) and one recognized by CD4⁺ T cells have been identified thus far (22).

The expression profile, its role in tumor progression and its immunogenicity, make CEA an attractive target for immunotherapeutic purposes and worthwhile additional efforts for T-cell epitope identification.

By using a combination of bioinformatics and cellular approaches, we have identified an immunodominant CEA epitope recognized by CD4⁺ T cells from healthy donors and colon cancer patients in association with 7 HLA-DR alleles. We show here that the identified sequence contains a naturally processed epitope(s) and that CD4⁺ T cells specific for this sequence do not cross-react with analogue sequences, present on the homologous CD66 proteins, and potentially presented by normal hematopoietic cells.

MATERIALS AND METHODS

T-Cell Epitope Prediction and Peptide Synthesis. We selected 11 sequences of the CEA protein for peptide synthesis, based on the TEPITOPE algorithm (23). We set the prediction threshold (*i.e.*, the percentage of best scoring natural peptides) at 5%, and we selected the sequences predicted to bind at least 40% of the HLA-DR molecules included in the software. The selected sequences were: p1-CEA_{13–25} (IPWQRLLLTASLL), p2-CEA_{51–63} (VLLL VHNLPQHFLF), p3-CEA_{99–111} (IYPNASLLIQN), p4-CEA_{117–129} (TGFYTLHVIKSDL), p5-CEA_{177–189}/CEA_{355–367} (LWWVNNQSLPVSP), p6-CEA_{425–437} (TYRPGVNLDSLSC), p7-CEA_{447–459} (YSWLIDGNIQQHT), p8-CEA_{533–545} (LWWVNGQSLPVSP), p9-CEA_{568–582} (AYVCGIQNS-VSANRS), p10-CEA_{652–666} (TYACFVSNLATGRNN), and p11-CEA_{666–678} (NSIVKSTITVSASG). The sequences were synthesized by manual parallel synthesis as described (24). Sequence corresponding to analogue sequence

Received 7/27/03; revised 8/26/03; accepted 9/23/03.

Grant support: Italian Association for Cancer Research, European Community [QLK2-CT2000-00470 (to M. P. P.) and QLK2-CT2001-01205 (to P. D. and G. C.)], Italian Ministry of University and Scientific Research, and Italian Ministry of Health, Compagnia di San Paolo.

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⁶The abbreviations used are: APC, antigen-presenting cell; CEA, carcinoembryonic antigen; PBMC, peripheral blood mononuclear cell; LCL, EBV-transformed lymphoblastoid cell lines; mAb, monoclonal antibody; TCR, T-cell receptor; ATCC, American Type Culture Collection.

CD66a₁₇₇₋₁₈₉ (LWWINNQLSPVSP) was also synthesized; analogue sequence CD66b/c₁₇₇₋₁₈₉ was equal to p8-CEA₅₃₃₋₅₄₅. The peptide purity was verified by reverse-phase high-performance liquid chromatography and electron spray mass spectrometry. The synthetic peptides were lyophilized, reconstituted in DMSO at 10 mg/ml, and diluted in RPMI 1640 (Life Technologies, Inc., Grand Island, NY), as needed.

Subjects and Cells. PBMCs were obtained from four healthy subjects (donors 1–4) and two colon carcinoma patients (donor/patients 5 and 6). The Institutional Ethics Committee approved the study protocol, and informed consent was obtained from all healthy subjects and patients before blood sampling. The colon carcinoma Lovo and the gastric carcinoma Kato III cell lines were purchased from ATCC (Manassas, VA). The LCLs used were: Com and Bor (established in our laboratory); PMH-161, BM21, LB, TEM, and OLL (kindly provided by K. Fleischhauer; Hospital San Raffaele, Milan, Italy); Leis-NIH (a generous gift from F. Marincola, National Institutes of Health, Bethesda, MD); and Pitout (purchased from the European Collection of Cell Culture, Salisbury, United Kingdom). The HLA-DR types of donors and tumor cell lines were identified by molecular or serological typing and are reported in Table 1.

Western Blot Analysis. Two million colon cancer cells were washed twice with Tris-buffered saline and lysed with NP40 (final concentration, 1%) for 30 min at 4°C in the presence of protease inhibitors. The sample was electrophoresed in a 7% polyacrylamide gel and then transferred to nitrocellulose paper. Immunoblotting was performed as described by Towbin *et al.* (25). The blot was incubated with 5% nonfat dry milk in Tris-buffered saline buffer, then with 1:1000 dilution of anti-CEA IgG antibody conjugated to peroxidase (a generous gift from Dr. C. Rosa, Sorin, Saluggia, Italy) for 1 h and processed for enhanced chemiluminescence according to the supplier's instructions.

Propagation of CD4⁺ T Cells. Synthetic peptides corresponding to the CEA sequences were pooled and used to stimulate the PBMCs from the different donors. Briefly, 20 × 10⁶ PBMCs were cultured for 7 days in RPMI 1640 (Life Technologies, Inc.) supplemented with heat-inactivated human serum (10%; Technogenetics, Milan, Italy), L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (50 μg/ml; Biowhittaker, Walkersville, MD) containing the CEA pool (1 μg/ml of each peptide). The reactive lymphoblasts were isolated on a Percoll gradient (26), expanded in interleukin 2 (10 units/ml) containing medium (T cell growth factor, Lymphocult; Biotest Diagnostic Inc., Dreieich, Germany), and restimulated at weekly intervals with the same amount of peptides plus irradiated (4000 rad) autologous PBMCs as APCs. CD4⁺ T-cell clones were obtained by limiting dilution from the polyclonal line from donors 2 and 4 and donor/patient 5, as described (27).

Flow Cytometry. Cytofluorimetric analyses were performed on a FAC-StarPlus (Becton Dickinson, Sunnyvale, CA). We used the following mAbs: anti-CD4-PE and anti-CD8-FITC (Becton Dickinson) and anti-DR (D1.12 hybridoma; ATCC). FITC-rabbit antimouse immunoglobulin antibody (DAKO A/S, Glostrup, Denmark) was used as second-step reagent in indirect immunofluorescence stainings.

Proliferation Assay. CD4⁺ T cells and autologous irradiated PBMCs or HLA-DR-matched LCL as APCs were diluted at a 1:10 or 1:5 ratio, respec-

tively, and used as described (28). Stimulants were CEA pool (0.5, 1, and 5 μg/ml), each peptide (10 μg/ml), purified CEA proteins (20 μg/ml; BiosPacific, Emeryville, CA; Calbiochem, Darmstadt, Germany), or normal human immunoglobulin (20 μg/ml; Venimmun N; Aventis Behring). Triplicate wells with CD4⁺ T cells alone and APCs alone were used as controls. Three wells with CD4⁺ T cells plus APCs did not receive any stimulus to determine the basal growth rate. In inhibition experiments, mAb D1.12 or an isotype-matched irrelevant mAb (W6/32; ATCC) was added at 25–50 μg/ml. After 48 h, the cultures were pulsed for 16 h with [³H]TdR (1 mCi/well, 6.7 Ci/mol; Amersham Corp., Milan, Italy). The cells were collected with a FilterMate Universal Harvester (Packard) in specific plates (Unifilter GF/C; Packard), and the thymidine incorporated was measured in a liquid scintillation counter (TopCount NXT; Packard). In competition assays, increasing amounts of competitor peptides (LWWINNQLSPVSP or LWVWNGQSLPVSP; 1, 5, 10, and 50 μg/ml) were preincubated with PBMCs as APCs for 2 h, and then CD4⁺ T cells were added in the presence of a suboptimal concentration of p5 (5 μg/ml).

Cytotoxicity Assay. CD4⁺ T cells were tested for specific lytic activity in a standard 4-h ⁵¹Cr release assay as described (28). The following targets were used: Lovo and Kato III cell lines and unpulsed and p5-pulsed LCL (LB). To allow the expression of MHC class II molecules, tumor cells were cultured for 48 h in the presence of IFN-γ (1000 units/ml; R&D Systems, Minneapolis, MN).

CD4⁺ T-Cell Stimulation Assay. Autologous APCs pulsed with purified CEA protein (20 μg/ml) or human IgG (20 μg/ml) were tested for their ability to induce the production of IFN-γ by peptide-specific CD4⁺ T cells, after 24–48 h of incubation, using a standard ELISA (Biosource Europe, SA, Nivelles, Belgium), following the manufacturer's instructions.

RESULTS

We selected 11 CEA sequences, based on prediction by TEPITOPE, and used a pool of the 11 peptides (CEA pool) to propagate polyclonal T-cell lines from four healthy donors (1–4) and two colon carcinoma patients (5 and 6; Table 1). Total PBMCs were stimulated with CEA pool for 7 days, and activated cells were expanded in the presence of interleukin 2 and restimulated weekly with irradiated peptide-pulsed autologous PBMCs as APCs. For all donors, after two or three cycles of stimulation, we obtained lines that contained only CD4⁺ T cells (data not shown).

Recognition of p5 by Long-Term Polyclonal CD4⁺ T-Cell Lines. We first tested the response of CD4⁺ T cells from all donors to the pool of CEA peptides, and then we determined the epitope repertoire of the CEA pool-specific CD4⁺ T cells by testing, at different weeks of propagation of the culture, their proliferative response to each single peptide forming the CEA pool. At the beginning of the culture, CD4⁺ T cells from the different donors had a larger repertoire; however, after a variable number of weeks of propagation (2–4), depending on the donor, all CD4⁺ T-cell lines strongly and uniquely recognized the CEA sequence repeated at positions 177–189 and 355–367 (p5; Table 2), thus identifying p5 as an immunodominant sequence.

HLA-DR Restriction of p5-specific CD4⁺ T Cells. HLA-DR restriction of p5-specific CD4⁺ T cells was first verified *in vitro* by inhibition of their proliferation to the peptide in the presence of an anti-HLA-DR antibody in the culture (data not shown). To identify the HLA-DR restricting alleles for the CEA immunodominant sequence, CD4⁺ T cells from all donors were challenged in microproliferation assays with LCL, expressing each of the two HLA-DRB1 alleles of the donor, pulsed with p5 (Fig. 1). p5 was recognized in association with HLA-DR*13 by donor 1, HLA-DR*03 and HLA-DR*07 by donor 2, HLA-DR*07 and HLA-DR*14 by donor 3, HLA-DR*1101 and HLA-DR*1104 by donor 4, HLA-DR*0405 and HLA-DR*14 by donor/patient 5, and HLA-DR*1104 by donor/patient 6. We also obtained several p5-specific CD4⁺ T-cell clones from donor 2 that were either HLA-DR3 or HLA-DR7 restricted, from donor 4 either

Table 1 HLA-DRB1 type of donors and cells used in this study

	Cell type	HLA-DRB1
Donors		
1	PBMC	DR*08, DR*13
2	PBMC	DR*03, DR*07
3	PBMC	DR*07, DR*14
4	PBMC	DR*1101, DR*1104
5	PBMC	DR*0405, DR*14
6	PBMC	DR*0403, DR*1104
Cells		
Lovo	Colon carcinoma	DR13
Kato III	Gastric carcinoma	DR2, DR8
LB	EBV-LCL	DR13
Pitout	EBV-LCL	DR*0701
Com	EBV-LCL	DR*0301
BM21	EBV-LCL	DR*1101
TEM	EBV-LCL	DR*14
Bor	EBV-LCL	DR*1104
OLL	EBV-LCL	DR8
Leis NIH	EBV-LCL	DR*0403, DR*13
PMH-161	EBV-LCL	DR*0405

Table 2 Epitope repertoire of CD4⁺ T cells from six donors propagated *in vitro* with CEA pool

Polyclonal CD4⁺ T-cell lines from the six donors (1–6), propagated *in vitro* with the CEA pool, were tested with each single peptide (10 μg/ml) forming the pool in 2-day microproliferation assays. The data, representative of several experiments, are expressed as cpm × 10⁻³ and are means of triplicate determination ± SD. Responses significantly higher than the blanks (*i.e.*, the basal level of proliferation of CD4⁺ T cells in the presence of autologous PBMCs as APCs: B + APC) were determined by unpaired, one-tailed Student's *t* test and indicated as ^a *P* < 0.001.

	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6
B + APC	0.1 ± 0	8.5 ± 1.4	10 ± 0.2	11 ± 1.5	5.9 ± 0.9	1 ± 0.1
p1	0.2 ± 0	5.9 ± 0.4	11.5 ± 1.3	9.4 ± 1.6	6.9 ± 0.6	0.3 ± 0.1
p2	0 ± 0	3.6 ± 0.8	6 ± 0.6	10.9 ± 1.5	6.7 ± 0.1	0.1 ± 0
p3	0.2 ± 0	10 ± 0.8	9.5 ± 2.6	10.3 ± 1	6.3 ± 0.2	1.4 ± 0
p4	0 ± 0	6.3 ± 1	11.3 ± 0.8	12.4 ± 8.4	6.8 ± 0.8	0.6 ± 0.1
p5	45 ± 3 ^a	30 ± 4 ^a	87 ± 7 ^a	45 ± 4 ^a	18 ± 1 ^a	6 ± 1 ^a
p6	1 ± 0	8.2 ± 0	9.4 ± 1.2	10 ± 1.6	6 ± 2	0.9 ± 0.1
p7	0.1 ± 0	5 ± 0.8	7 ± 0.6	10 ± 1	4.5 ± 0.7	0.1 ± 0
p8	0.7 ± 0	4.7 ± 0.5	15 ± 3	11 ± 7	5 ± 0.2	0.2 ± 0.1
p9	0.1 ± 0	8.6 ± 2	9 ± 0.5	7 ± 1.5	5 ± 3	0.2 ± 0.1
p10	0.2 ± 0	7.4 ± 3	11 ± 1	15 ± 6	4.6 ± 3	0.9 ± 0.1
p11	0.3 ± 0	8 ± 0.3	9.8 ± 0.4	11 ± 1.7	6 ± 2	0.9 ± 0.3

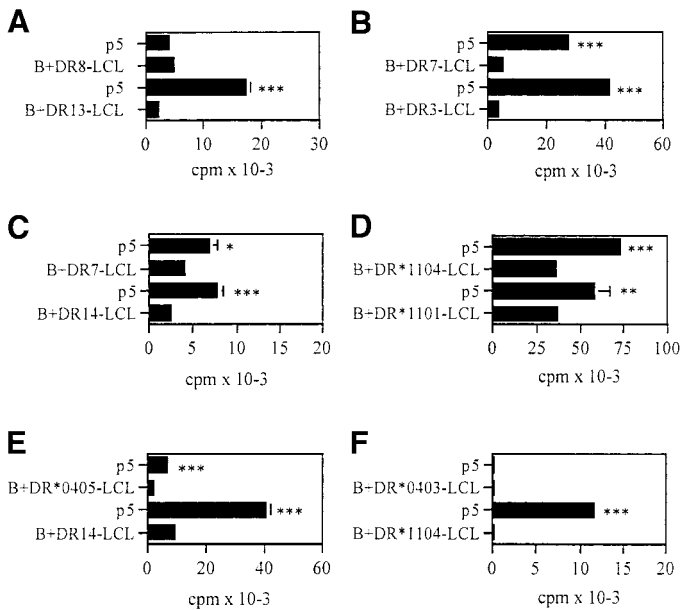


Fig. 1. HLA-DR restriction of CD4⁺ T cells specific for immunodominant sequence p5. CD4⁺ T cells from the six donors (1–6) were challenged in 2-day microproliferation assays with p5, in the presence of LCLs expressing each of the HLA-DRB1 alleles of the donor. The blanks (*i.e.*, the basal level of proliferation of CD4⁺ T cells in the presence of the LCLs) are expressed as B+LCL. The data are means of triplicate determination ± SD and are representative of several experiments. A, donor 1 (HLA-DR8 and -DR13). B, donor 2 (HLA-DR3 and -DR7). C, donor 3 (HLA-DR7 and -DR14). D, donor 4 (HLA-DR*1101 and -DR*1104). E, donor/patient 5 (HLA-DR*0405 and -DR14). F, donor/patient 6 (HLA-DR*0403 and -DR*1104). Responses significantly higher than the blanks are indicated as: *, *P* < 0.05; **, 0.001 < *P* < 0.05; ***, *P* < 0.001 (determined by unpaired, one-tailed Student's *t* test).

HLA-DR*1101 or HLA-DR*1104, and from donor/patient 5 either HLA-DR*0405 or HLA-DR14 (data not shown). We cannot exclude that HLA-DRB3 and HLA-DRB4 molecules in addition to HLA-DRB1 can also present the CEA immunodominant sequence to the polyclonal CD4⁺ T-cell lines.

Recognition of Native CEA by p5-specific CD4⁺ T Cells. To verify whether p5 contains a naturally processed epitope(s), we tested the recognition of the native CEA protein both after processing and presentation by autologous APC after phagocytosis of purified CEA protein and directly by recognition of carcinoma cells expressing endogenous CEA and MHC class II molecules (Figs. 2 and 3). CD4⁺ T-cell lines from five donors (1–5) were challenged with autologous APC pulsed with the CEA protein and assayed for [³H]thymidine incorporation (Fig. 2A) and/or IFN-γ release (Fig. 2B). CD4⁺ T cells from all tested donors strongly and significantly recognized the CEA protein whereas they did not recognize normal human IgG, demon-

strating their CEA specificity. Because CD4⁺ T cells produce large amounts of INF-γ, we tested their lytic activity. As shown in Fig. 3, CD4⁺ T cells that recognized p5 in association with HLA-DR13 (Fig. 1A), killed DR13-LCL pulsed with p5 and, importantly, Lovo cells expressing CEA and HLA-DR13, whereas they did not kill Kato III cells, expressing CEA and unrelated HLA-DR alleles, or unpulsed HLA-DR13-LCL (Fig. 3A). The levels of expression of CEA (Fig. 3B) as well as of surface MHC class II molecules (Fig. 3C), after a 48-h culture in the presence of INF-γ, by tumor cells are also shown.

p5-specific CD4⁺ T Cells Do Not Cross-React with Analogue Self-Sequences Present on Homologous Proteins of the CD66 Family. Because CEA, or CD66e, belongs to a family of highly homologous proteins that are expressed at high levels in normal hematopoietic cells, we verified the sequence similarity of p5 among the different CD66 molecules. Two sequences that differ from p5 for

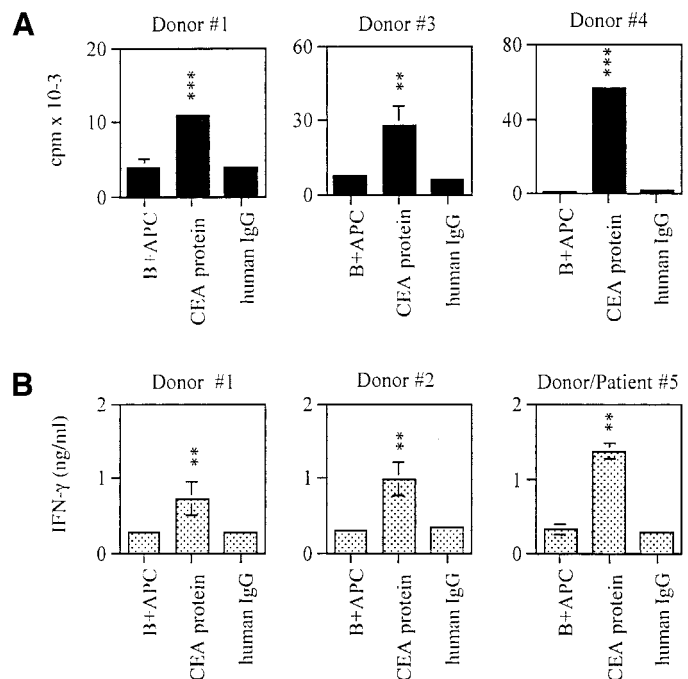


Fig. 2. p5-specific CD4⁺ T cells recognize the native CEA protein. CD4⁺ T cells from five donors (1–5) were challenged *in vitro* with the CEA protein (20 μg/ml) or human IgG (20 μg/ml) in the presence of autologous APCs and tested in 2-day microproliferation assays (A) and/or IFN-γ release (B). The blanks (*i.e.*, the basal level of proliferation or IFN-γ release of CD4⁺ T cells in the presence of autologous PBMCs as APCs) are expressed as B+APC. The data are means of triplicate determination ± SD and are representative of at least two experiments for each CD4⁺ T-cell line. Responses significantly higher than the blanks are indicated as: **, 0.001 < *P* < 0.05; ***, *P* < 0.001 (determined by unpaired, one-tailed Student's *t* test).

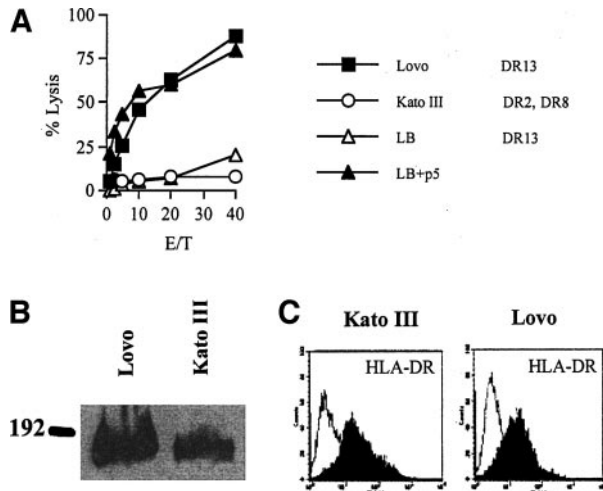


Fig. 3. Recognition of colon carcinoma cells by p5-specific CD4⁺ cytotoxic T cells. *A*, cytolytic activity, measured in a ⁵¹Cr release assay, of HLA-DR13-restricted p5-specific CD4⁺ T cells from donor 1, against HLA-DR-matched or unmatched carcinoma cells expressing CEA. The targets used and their HLA-DR types are shown. The results are representative of several experiments. *B*, Western blot analysis of the expression of CEA by tumor cell lines (Lovo and Kato III) used as targets. *C*, cytofluorimetric analysis for surface MHC class II expression in tumor cells used as targets. *Filled histograms*, stained sample; *open histograms*, background staining obtained with FITC-conjugated second-step reagent only.

only one amino acid (substitutions I→V and G→N, respectively) were found. The first analogue (LWWINNGQSLPVSP) is present in the CD66a molecule at residues 177–189. The second (LWVWNGQSLPVSP) is present in the CD66b and CD66c molecules, at residues 177–189. This latter sequence is also present in the CEA or CD66e molecule at residues 533–545 (p8) and is comprised in the CEA pool used to propagate the CD4⁺ T-cell lines and never elicited proliferative activity in any donor. Fig. 4 shows the results of the experiments of cross-reactivity of p5-specific CD4⁺ T cells from donor 1 and donor/patient 5 in the presence of the two CD66 analogues. CD4⁺ T cells proliferated in the presence of p5 but not in the presence of the analogues, demonstrating that recognition of p5 is, indeed, CEA specific (Fig. 4, *A* and *B*). To discriminate whether the lack of cross-recognition was because of poor binding to the HLA-DR molecules or to lack of TCR stimulation, we performed competition experiments in which APCs prepulsed with increasing amounts of competitor peptide (1, 5, 10, and 50 μg/ml) were used in microproliferation assays to stimulate CD4⁺ T cells from donor 1 in response to a suboptimal dose (5 μg/ml) of p5 (Fig. 4C). The response of CD4⁺ T cells to p5 decreased, in a dose-dependent manner, in the presence of peptide CD66a_{177–189}, whereas it was only marginally affected in the presence of peptide CD66b/c_{177–189} (p8). These results demonstrate that peptide CD66a_{177–189} competes with p5 for binding to the HLA-DR molecules, but the MHC-peptide complex is not recognized by the TCR of p5-specific CD4⁺ T cells, whereas peptide CD66b/c_{177–189} (p8) seems to be a poor binder.

DISCUSSION

In this study, we used a combined approach of bioinformatics, followed by *in vitro* validation with biological assays to identify the amino acid sequence repeated at residues 177–186 and 355–367 (p5) on the CEA protein as a strongly immunogenic and immunodominant region that contains a naturally processed epitope(s).

The ability of the immune system to focus on a selected number of epitopes of a complex antigen is a distinctive feature of most T-cell immune responses and it is termed “immunodominance.” The iden-

tification of immunodominant epitopes on tumor antigens is of particular importance for vaccine development to increase the number of patients eligible for therapy. Immunodominance may be dictated by antigen processing mechanisms, which may vary in different cell types, by intermolecular competition for MHC binding, by HLA-DM molecules, and by the existence of a biased T-cell repertoire (29, 30). In the case of the CEA, the epitope immunodominance may also be related to the high degree of glycosylation of the molecule. Indeed, CEA is a glycoprotein with a molecular weight of M_r 180,000 that comprises 60% carbohydrate with 28 potential glycosylation sites. Because of this, available linear sequences potentially able to form CD4 epitopes are probably significantly reduced compared with nonglycosylated proteins. Indeed, we recently identified four immunodominant regions on the tumor-specific antigen MAGE-3, which is a nonglycosylated protein with a molecular weight of M_r 74,000 (31). We cannot exclude that other mechanisms besides glycosylation may account for the different characteristics in the T-helper response seen between the two tumor antigens. Nonetheless, using different experimental approaches, other immunodominant as well as subdominant linear and glycopeptidic CEA epitopes able to activate CD4⁺ T-cell responses might also be found. The existence of MHC class II-restricted immunogenic glycopeptides has been documented previously for the melanoma antigen tyrosinase (32) and the tumor antigen MUC1 (33).

Recently, a CEA-helper epitope at residues 653–667, able to induce proliferation of CD4⁺ T cells in association with HLA-DR4, -DR7, and -DR9, was identified (22). This sequence largely overlaps p10-CEA_{652–666} that is comprised in the CEA pool used in our study and that never elicited CD4⁺ T-cell responses from any subject studied. This discrepancy may be explained by the strong immunodominance of p5, which was not tested by Kobayashi *et al.* (22), and it suggests that sequence CEA_{653–667} may contain a subdominant epitope(s).

We showed that p5 contains a naturally processed epitope(s) and the

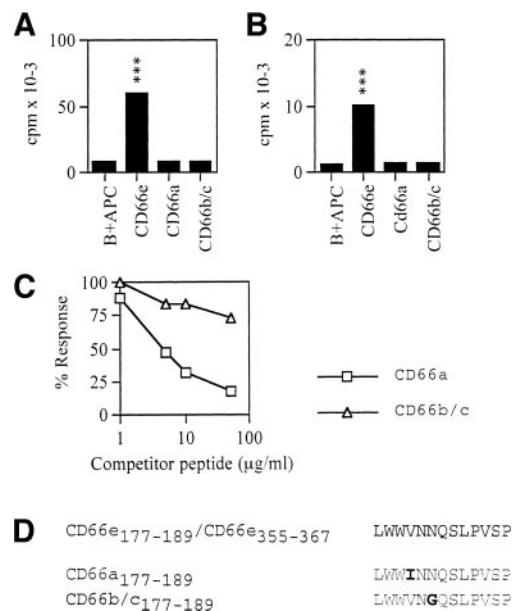


Fig. 4. Cross-reactivity experiments of p5-specific CD4⁺ T cells for CD66 analogue sequences. CD4⁺ T cells from donor 1 (*A*) and donor/patient 5 (*B*) were challenged with autologous PBMCs as APCs, pulsed with p5 or synthetic peptides corresponding to the analogues belonging to different members of the CD66 family. The data are representative of at least two experiments for each donor. Responses significantly higher than the blanks are indicated as: ***, $P < 0.001$ (determined by unpaired, one-tailed Student's *t* test). *C*, competition experiments: APCs from donor 1 were pulsed for 2 h with increasing amounts of competitor peptides (1, 5, 10, and 50 μg/ml), and CD4⁺ T cells were then added in the presence of suboptimal concentration of p5. *E*, alignment of the CD66 analogue sequences.

native sequence(s) could be produced through both the exogenous and the endogenous processing pathways. In fact, native CEA_{177–186/355–367} was produced and presented by APCs after uptake and processing of the soluble protein and directly by HLA-DR molecules on tumor cells. In this case, the epitope may be produced either in the cytoplasm and reach the endosomal/lysosomal compartment through a leakage from the endogenous pathway or directly in the endosomal/lysosomal compartment from CEA recycling from the plasma membrane.

A fundamental feature of the epitope(s) identified in our study is the strict specificity for CEA. CEA belongs, in fact, to a family of highly homologous proteins, some of which are expressed in normal cells (*i.e.*, granulocytes, monocytes, dendritic cells, and activated T and B lymphocytes). Therefore, the use of CEA as vaccine has the potential to induce autoimmune responses against normal hematopoietic cells. The generation and availability of immunogenic self-epitopes during ontogeny impinge directly on the induction of central and peripheral tolerance and the consequent generation of a responsive T-cell repertoire. The CEA, although not expressed directly into primary or secondary lymphoid organs, share extensive sequence homology with members of the CD66 family expressed within the lymphoid tissue. CD4⁺ T cells are, thus, exposed to several CEA analogue sequences during ontogenesis. This process may well lead to the induction of tolerance against these epitopes, resulting in a marked shaping of the CEA-specific peripheral CD4⁺ T-cell repertoire toward a limited number of CEA epitopes that are not encountered during ontogenesis. We showed that, although the immunodominant CEA epitope differs from the analogue self-sequences for only one amino acid, its recognition is very selective. Indeed, one (CD66b/c_{177–189}) of the two competitor peptides bound the MHC class II molecules very poorly. The other (CD66a_{177–189}) bound the MHC complex at high affinity, however, the MHC-peptide complex could not be recognized by the TCR of the p5-specific CD4⁺ T cells.

CD4⁺ T cells specific for the analogues have been probably deleted either in the thymus or in the periphery. On the contrary, the repertoire of p5-specific CD4⁺ T cells is present both in normal donors and colon cancer patients, and the TCR of these cells does not allow self-antigens recognition through molecular mimicry, resulting in a repertoire of CD4⁺ T cells specific for CEA. Indeed, the lack of cross-reactivity of p5-specific CD4⁺ T cells for the analogues may be considered an additional mechanism of self-tolerance. A lack of cross-reactivity with homologous self-proteins (Kallikrein) by PSA cytotoxic T cells was demonstrated previously (34), pointing to possible similar mechanisms of immune tolerance in both models.

Recently, the induction of an anti-CEA response in the absence of autoimmunity was demonstrated in CEA transgenic mice vaccinated with recombinant vaccinia virus-expressing CEA (35). Nonetheless, it will be important to address the question of the possible induction of autoimmunity in clinical trial settings.

In conclusion, the strong immunodominance and the lack of cross-reactivity *in vitro* of CD4⁺ T cells for analogue self-sequences, potentially expressed on hematopoietic cells, make the identified immunodominant CEA sequence an excellent candidate for peptide-based immunotherapeutic intervention in patients bearing CEA-positive tumors. Moreover, it represents a valuable tool to characterize the natural anti-CEA CD4⁺ T-cell response as well as to monitor the anti-CEA CD4⁺ T-cell response before and after vaccination using different strategies (18, 36–38).

ACKNOWLEDGMENTS

We thank Angela Bachi for mass spectrometry analyses and Matteo Bellone and Angelo Manfredi for critical reading of this manuscript.

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