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ORIGINAL ARTICLE

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HLA-A3 restricted mutant ras specific cytotoxic T-lymphocytes induced by vaccination with T-helper epitopes

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Abstract Cytotoxic T-lymphocytes are one of the most important elements of the antitumor defense. Stimulation of cytotoxic T-lymphocytes outgrowth after immunization with mutant ras peptides is a desired goal since these cells may kill tumor cells in vivo. In this study we tested responding peripheral mononuclear cells from a patient with pancreatic adenocarcinoma who had received intradermal peptide vaccination with a mixture of 17-mer mutant ras peptides and granulocyte-macrophage colony-stimulating factor as an adjuvant. Responding peripheral T-cells were cloned by limiting dilution and several $CD8^+$ cytotoxic T-lymphocytes, specific for the K-*RAS* 12-Cys mutation were obtained. By using a panel of nonamer peptides containing the 12-Cys mutation and covering position 4–21 in the ras molecule, the 9-mer peptide which was actually recognized by the cytotoxic T-lymphocytes could be identified. HLA-A*0302 could be identified as the antigen-presenting molecule, and the amino acid sequence of the T-cell epitope carries the previously identified HLA-A*0302 binding motif. The nonamer peptide was contained within the vaccine peptide originally used for intradermal immunization of the patient. The cytotoxic T-lymphocytes were capable of killing target cells expressing HLA-A*0302 that coexpressed the K-*RAS* 12-Cys mutation after transfection. These data demonstrate that the peptide used for vaccination (17-mer) is processed and presented in vivo, and that generation of cytotoxic T-lymphocytes by vaccination with T-helper epitopes may be important for further development of specific immunotherapy of cancer patients.

Keywords Cytotoxic T lymphocytes · p21 ras · Peptide vaccination · Pancreatic cancer · Granulocyte-macrophage colony-stimulating factor

Abbreviations *APC:* Antigen-presenting cell · *B-LCL:* B-Lymphoblastoid cell line · *CTL:* Cytotoxic T lymphocyte · *DC:* Dendritic cell · *GM-CSF:* Granulocyte-macrophage colony-stimulating factor · *HLA:* Human leukocyte antigen · *mAb:* Monoclonal antibody · *MHC:* Histocompatibility complex · *PBMC:* Peripheral mononuclear cell

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Introduction

The potential role of cytotoxic T lymphocytes (CTL) in resistance to malignant diseases has been reemphasized

in recent years. Antigen-specific CTLs are recognized as a possible defense mechanism in malignant melanoma [1, 2, 3] and have also recently been described in cervical carcinoma [4] and for other solid epithelial tumors such as breast, lung, and intestinal tumors [5]. T lymphocytes can recognize antigens in association with major histocompatibility complex (MHC) class I or class II molecules [6]. These surface antigens are composed of short peptides derived from degraded intracellular proteins [7]. $CD8^+$ T lymphocytes recognize peptides (8–10 residues) bound to MHC class I molecules and are associated with cytotoxic activity [8, 9]. The $CD4^+$ T lymphocytes recognize peptides (13–18 residues) presented by MHC class II molecules [10] and are involved in immunoregulation through cytokine secretion [11]. For CTL activation to occur not only must antigens be presented as peptide fragments on MHC products, but the antigen-MHC complexes must also be introduced on cells with the requisite accessory functions that lead to T cell growth and cytolytic activity. Dendritic cells (DCs) are specialized antigen-presenting cells (APCs) for the initiation of such T cell dependent immune responses [12].

The ras p21 proto-oncogenes encode highly conserved intracellular proteins that are involved in cell proliferation and differentiation. Mutations in the *RAS* proto-oncogenes are among the most frequent alterations found in human tumors and occur at a limited number of residues: 12, 13, or 61 [13]. Mutations in K-*RAS* genes have been reported in a variety of human malignancies and are detected in about 90% of pancreatic adenocarcinomas [14]. Mutated ras proteins can be considered tumor specific and represent an attractive target for a T-cell mediated response since they are not expressed on normal cells and are necessary to maintain the neoplastic phenotype [15]. Mutated ras proteins or corresponding peptide sequences have been shown to be immunogenic in healthy individuals and cancer patients [16, 17, 18, 19, 20, 21]. Peptide-specific T cell responses against mutant ras can also be induced in vivo in cancer patients by peptide vaccination [22, 23, 24]. These peptides which are 13–17 amino acids in length, represent natural ras epitopes, and are designed primarily to induce CD4 T helper-specific immune responses, but also $CD8^+$ T cells specific for nested epitopes encompassing the ras mutation have been described [25, 26]. Both T cell subsets can lyse autologous tumor cells or human leukocyte antigen (HLA) matched cancer cell lines expressing the corresponding K-*RAS* mutation, demonstrating that relevant peptide epitopes are generated by endogenous processing of mutant p21 ras in the tumor cells. Thus, mutated p21 ras proteins may bear unique antigenic determinants for immune recognition by T cells and such T cells can be selectively expanded in cancer patients after vaccination.

Stimulation of CTL outgrowth after immunization with mutant ras peptides is a desired goal, since these CTLs may kill tumor cells. Here we used responding peripheral mononuclear cells (PBMCs) from a pancreatic carcinoma patient who had received intradermal ras peptide vaccination with granulocyte-macrophage colony-stimulating factor (GM-CSF) as an adjuvant [24]. Responding peripheral T cells were cloned by limiting dilution, and several $CD4^+$ and $CD8^+$ T cell clones specific for the 12-Cys mutation were obtained. We report the identification of a mutant ras specific $CD8^+$ T cell peptide epitope which was found to be HLA-A*0302 restricted. The peptide epitope reflecting the 12-Cys mutation was determined to be a 9-mer sequence nested within the longer 17-mer vaccine peptide originally used for vaccination and reflecting the previously identified HLA-A*0302 motif [27]. The CTLs were capable of killing target cells expressing HLA-A*0302 that expressed the K-*RAS* 12-Cys mutation after transfection. These studies illustrate and further support a potentially important biological concept of overlapping or nested $CD4^+$ and $CD8^+$ T cell epitopes within a single peptide immunogen. Stimulation and activation of such CTLs in vivo by ras peptide vaccination may be crucial for further vaccine development and may be of importance for the demonstration of objective tumor responses in cancer patients.

Materials and methods

T cell donor

The woman, 53 years old at diagnosis, had an unresectable adenocarcinoma of the pancreatic head. She was immunized intradermally with a mixture of 17-mer mutant ras peptides in combination with GM-CSF as previously described [24]. Briefly, the vaccination protocol consisted of weekly intradermal injections (for 4 weeks, and a booster vaccination in weeks 6 and 10) of 100 μg of each peptide (1 mg/ml) of a mixture of four mutant ras peptides (12-Asp, 12-Val, 12-Arg, 12-Cys) in 0.1 ml saline. Fifteen minutes prior to peptide injection 40 μg recombinant human GM-CSF (Leucomax, Schering-Plough, Ireland) in 0.1 ml saline was administered by intradermal injection at the same site. After four consecutive peptide injections the patient developed both a proliferative T-cell response assessed in peripheral blood and a positive delayed-type hypersensitivity reaction as evidence of successful immunization. The HLA type of the patient was HLA-A*0302,6801; HLA-B*4402,5001; HLA-Cw*0501,0602. The K-*RAS* mutation Gly12→Val was identified in DNA from formalin-fixed paraffin-embedded tumor tissue using a highly sensitive technique [28].

Cells and media

PBMCs were prepared by centrifugation over Lymphoprep (Amersham, Oslo, Norway). Autologous B-lymphoblastoid cell lines (B-LCLs) were generated by Epstein-Barr virus transformation of B cells from the patient. B-LCLs used as APC were obtained from the International Histocompatibility Workshop (IHW) cell collection at the European Collection of Cell Cultures. The HLA profiles of the different cell lines used are given in Table 2. All cultures were grown in RPMI 1640 medium (Bio Whittaker, Walkersville, Md., USA) supplemented with 50 μg/ml gentamicin, 15% heat-inactivated human pool serum (T-cells) or 10% heat-inactivated fetal calf serum (Gibco, Paisley, UK; cell lines).

Peptides

Peptides were kindly provided by J.A. Eriksen (Hydro, Porsgrunn, Norway): peptides encompassing residues 5–21 of p21 ras,

Table 1 Amino acid sequence of the mutant ras peptides used for vaccination. The peptides consist of 17 amino acids and cover position 5–21 in the ras molecule

Sequence	Mutation
^{5}KLVVVGA *G* GVGKSALTI^{21}	12 Gly (normal ras sequence)
KLVVVGA *D* GVGKSALTI	12 Asp
KLVVVGA *V* GVGKSALTI	12 Val
KLVVVGA *R* GVGKSALTI	12 Arg
KLVVVGA *C* GVGKSALTI	12 Cys

KLVVVGAGGVGKSALTI (single-letter code) or with a Gly-to-Asp, -Val, -Arg, or -Cys substitution at residue 12 (Table 1) were synthesized and purified as previously described [11]. Nonamer peptides covering residues 4–20 of p21 ras and containing the 12-Gly, 12-Asp, 12-Val, 12-Arg, or 12-Cys substitution were additionally synthesized. The peptides were dissolved in sterile water before filter sterilization and stored in aliquots at –70°C.

Antibodies

The following monoclonal antibodies (mAbs) were used in blocking experiments: W6/32: anti-HLA class I (hybridoma cells from American Type Culture Collection, Rockville, Md., USA); B7/21: anti-HLA-DP (gift of Dr. F. Bach, University of Minnesota, Minn., USA); SPV-L3: anti-HLA-DQ (gift of Dr. H. Spits, Palo Alto, Calif., USA); B8/11: anti-HLA-DR (gift of B. Malissen, Marseilles, France). The anti-CD3 (clone UCHT1), anti-CD4 (RPA-T4) and anti-CD8 (RPA-T8) mAbs were purchased from Pharmingen (San Diego, Calif., USA).

Generation of T-cell clones

Responding PBMCs from the patient were thawed and plated at 1×10^6 cells per well in 24-well plates (Costar, Cambridge, Mass., USA) and stimulated with autologous, irradiated (30 Gy) PBMC (1×10^6) per well and the 12-Asp, 12-Val, 12-Arg, and 12-Cys (5–21) ras peptides at 25 μM of each together with 10 U/ml recombinant interleukin-2 (Amersham, Aylesbury, UK). Four days after stimulation the bulk culture was cloned by seeding five cells per well onto Terasaki plates (Nunc, Roskilde, Denmark) together with 100 U/ml recombinant interleukin-2 and autologous PBMCs that were pulsed for 3 h at 37°C with the 9-mer series of peptides and the residues 5–21 of p21 ras peptide with either a 12-Val, 12-Arg, or 12-Cys mutation (1 μM of each peptide) in x-vivo 10 medium (Bio-Whitaker, Walkersville, Md., USA) supplemented with 3 μg/ml β_2-microglobulin (Sigma-Aldrich Chemie, Steinheim, Germany) and 10 ng/ml tumor necrosis factor-α (Biosource International, Nivelles, Belgium), then washed once and irradiated (30 Gy) and used as feeder cells with 2×10^4 cells/well. After 10 days, T-cell clones were transferred onto flat-bottomed 96-well plates (Costar) with 1 μg/ml phytohemagglutinin (Wellcome, Dartford, UK), 100 U/ml recombinant interleukin-2 and allogeneic, irradiated (30 Gy) PBMC (15×10^4) per well as feeder cells. After 4 days T cell clones were transferred to 24-well plates with phytohemagglutinin/recombinant interleukin-2 and 1×10^6 allogeneic, irradiated PBMC as feeder cells and screened for peptide specificity after 7 days or after another restimulation.

Cytotoxicity assays

Cytotoxicity of the CD8$^+$ CTLs was measured in a standard 4-h ^{51}Cr-release assay. Labeling of 2×10^6 target cells in FCS and ^{51}Cr (7.5 Mbq; Laborel, Oslo, Norway) was performed in a total volume of 0.5 ml for 1 h at 37°C with gentle mixing every 15 min. Cells were washed three times in cold RPMI 1640 medium, counted, and seeded at 2×10^3 target cells in 96-well, U-bottomed microtiter plates (Costar). Target cells were pulsed with peptide for 1 h at 37°C in a volume of 100 μl Iscove's modified Dulbecco's medium (Bio-Whittaker, Europe) or transfected with- or without 12-Cys mRNA at concentrations as indicated [29]. The target cells were washed once in the microtiter plates and effector cells were added at different numbers as indicated, to a final volume of 200 μl in 15% human pool serum RPMI 1640. In blocking experiments chromium-labeled target cells were admixed in triplicate with mAbs in U-bottomed 96-well microtiter plates (Costar) and incubated for 60 min at 37°C before addition of T cells. Mabs were used at a final concentration of 10 μg/ml. In peptide competition experiments chromium-labeled target cells were pulsed with the stimulator/competitor peptide mixture at different concentrations as indicated for 1 h at 37°C in a CO_2 incubator. Then the peptide mixture was removed, and the cells were washed three times with serum-free Iscove's modified Dulbecco's medium before plating and addition of T cells. Maximum and spontaneous ^{51}Cr release of target cells was measured after incubation with 5% Triton X-100 (Sigma) or medium, respectively. Supernatants were harvested after 4 h incubation at 37°C, and radioactivity was measured in a Packard Topcount microplate scintillation counter (Meriden, Conn., USA). The percentage of specific chromium release was calculated by the formula: (experimental release – spontaneous release)/(maximum release – spontaneous release) ×100.

Results

Identification of the peptide epitope and HLA restriction of the CTL clone EG2.9

In the patient a peptide-specific T-cell response to all four mutant ras peptides contained in the vaccine was induced in PBMC after the fourth vaccination [24]. The proliferative T-cell response was detected in a standard 7-day proliferation assay. Responding T cells were cloned by limiting dilution from a bulk culture initiated by stimulation with the mixture of the four mutant ras peptides (Table 1), and several peptide-specific T-cell clones of both the CD4$^+$ and CD8$^+$ phenotype were obtained (data not shown).

Although the antigens and culture conditions used were not generally favorable for the generation of CTL responses, we decided to investigate whether some of the CD8$^+$ clones were directed against nested epitopes present in the vaccine peptides. CTL clone EG2.9 was chosen for further characterization and was found to be CD3$^+$, CD8$^+$, and T cell receptor α,β$^+$ (data not shown). To assess the peptide specificity of CTL clone EG2.9, a panel of nonamer peptides covering residues 4 to 20 of p21 ras and containing either the 12-Asp, 12-Val, 12-Arg, or 12-Cys substitution was used. The actual peptide epitope recognized by the CTL clone EG2.9 was the 12-Cys (8–16) peptide (Fig. 1A). When the homologous ras peptides covering residues 8–16 but containing the other frequent ras mutations or the normal ras sequence (either 12-Asp,12-Val, 12-Arg, or 12-Gly) were used to pulse the target cells, no specific killing was observed (Fig. 1B). Antibody-blocking studies revealed that CTL clone EG2.9 was HLA class-I restricted, since mAbs against HLA-A, HLA-B, and HLA-C but not HLA-DP, HLA-DQ, or HLA-DR abolished the response (Fig. 2). It

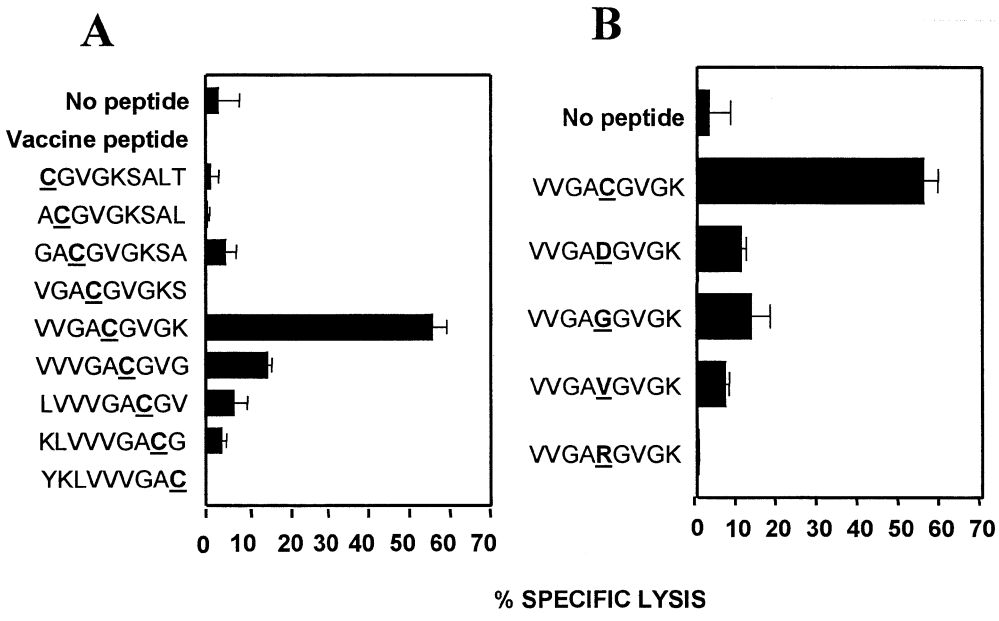

Fig. 1A, B Peptide specificity of CTL clone EG2.9. **A** By using a panel of nonamer peptides covering positions 4–20 in the ras molecule and containing the 12-Cys mutation it was possible to identify the 12-Cys (8–16) peptide as the stimulating antigen. Controls included the vaccine peptide and medium alone. Cytotoxicity was determined in a standard 4-h Cr-release assay at an effector-to-target ratio of 10/1. **B** To further show fine specificity of the CTL clone EG2.9 homologous ras peptides covering residues 8–16 but containing the other frequent ras mutations or the normal ras sequence were used for stimulation. This figure shows that only the 12-Cys (8–16) peptide stimulated the CTL

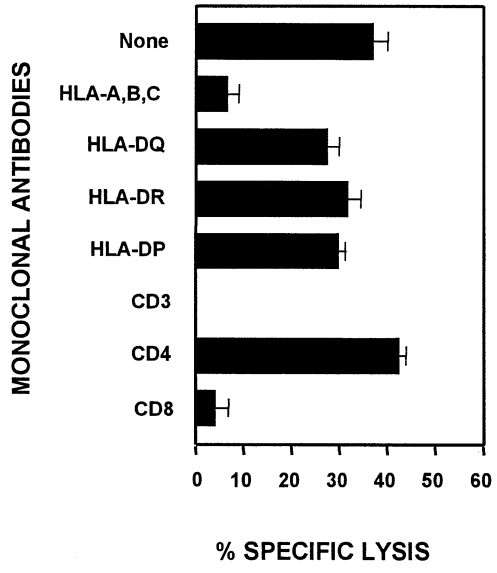

Fig. 2 Determination of cytolytic response of the CTL clone EG2.9 as HLA class I restricted and CD8+ mediated; mAbs directed against HLA class I (W6/32) and class II (B7/21: SPV-L3 and B8/11) molecules were used at a final concentration of 10 μg/ml. Results obtained with an effector-to-target ratio of 10/1 are shown. It is demonstrated that mAbs against HLA class I but not those against HLA-DP, HLA-DQ, or HLA-DR blocked the response. Furthermore, we demonstrate that the CTL clone EG2.9 is CD3+ and CD8+ since mAbs against the CD3 (clone UCHT1; 10 μg/ml) and the CD8 (clone RPA-T8; 10 μg/ml) but not against the CD4 (clone RPA-T4; 10 μg/ml) molecules ablated the cytolytic response

was also observed that the CTL clone EG2.9 was CD3+ and CD8+, since mAbs against CD3 and CD8 ablated the peptide-specific response whereas mAbs against CD4 did not (Fig. 2).

The patient carried the following HLA class I molecules: HLA-A*0302,6801; HLA-B*4402,5001, and HLA C*0501,0602. By employing a panel of homozygous B-LCLs as APCs HLA-A* 0302 was identified as the antigen-presenting molecule for CTL clone EG2.9 (Table 2).

Peptide sensitivity of CTL clone EG2.9

The sensitivity of CTL clone EG2.9 to exogenous peptide was examined in a dose-response experiment using peptide-loaded autologous B-LCLs as target cells (Fig. 3). Anti-ras CTL activity was detectable over a several-log range, with maximal lysis at 1 μM and half-maximal response at 0.005 μM peptide concentration.

Peptide competition with homologous ras peptides

The sequence of the ras protein around codon 12 contain binding motifs for HLA-A3 and have previously been screened for actual HLA-A3 binding by others [30]. They identified five ras peptides (one wild type, four mutated) that were able to bind the HLA-A3 molecule. All of these peptides had the same sequence (ras 8–16) as the nonamer peptide which we have identified as the stimulating peptide epitope for CTL clone EG2.9. The ras peptides were found to be moderate binders at 37°C, but the presence of β_2-microglobulin during peptide-pulsing allowed a significant increase in binding for four of five peptides (12-Val, 12-Ala, 12-Gly, and 12-Arg). Since CTL clone EG2.9 did not recognize any of the homologous ras 8–16 peptides, we wanted to investigate whether peptide stimulation could be blocked in a peptide-competition experiment. For this experiment the stimulating 12-Cys (8–16) peptide was titrated at various concentrations against the competitor peptide 12-Val (8–16) which was added at a fixed concentration. Both

Table 2 The ability of various B-LCLs to present the 12-Cys (8–16) peptide to the CTL EG2.9. Cromium-labeled B-LCLs were pulsed with the peptide (1 μM) and used as target cells at an effector-to-target ratio of 25:1 (*italics* HLA types shared with the patient, *% cytotoxicity* lysis of B-LCL with antigen minus lysis of B-LCL without antigen

B-LCL		HLA			EG2.9 % Cytotoxicity
Name	IHW no.	A*	B*	Cw*	
EG (autologous)	–	*0302, 6801*	*4402, 5001*	*0501, 0602*	75
L0081785	9018	0301, 2402	1801	*0501*	23
SCHU	9013	0301	0702	0702	43
CGM1	9207	*0302*, 2902	0801, 1402	0802	78
MOU	9050	2902	44031	1601	2
WT47	9063	3201	*4402*	*0501*	0
SPO010	9036	0201	*4402*	*0501*	0
HOR	9053	3303	44031	1403	0
PITOUT	9051	2902	44031	1601	0
IDF	9211	6601, 6901	18, 3801	1203	0
BM15	9040	0101	4901	0701	3
OMW	9058	0201	4501	1601	2
PFO4015	9088	0101	0801	0701	2
BOLETH	9031	0201	1501	0304	3
31227ABO	9061	0201	1801	0701	7
31708	9278	2301, 6802	1503, 35	0202, 0401	0
AMAI	9010	6802	5301	0401	11
GRC-187	9363	*68012,* 31012	1504, 3511	0303, 0304	2
LB LB-S	9289	*68011*	40011	0304	2
RSH	9021	3001, 6802	4201	1701	0
TO TO-PP	9218	3001, 6802	1302, 5101	*0602*, 15	0
SHJO SMJ	9239	2301, 2402	4201, *5001*	*0602*, 1701	0
MOU MANN	9050	2902	44031	1601	2

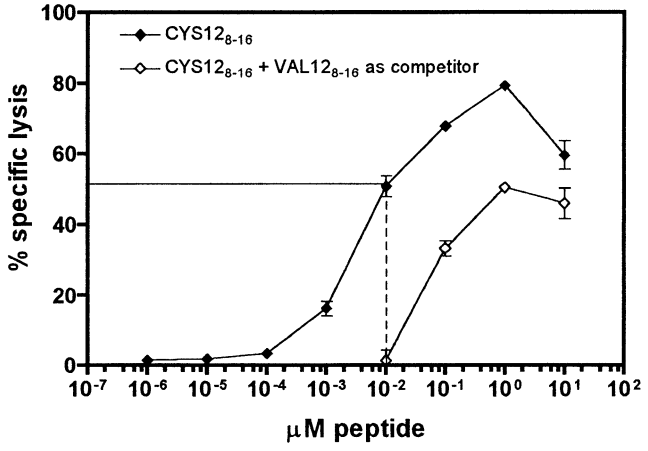

Fig. 3 Peptide sensitivity of CTL clone EG2.9 and inhibition of killing by a competitor peptide. The sensitivity of EG2.9 to exogenous peptide was examined in a dose-response experiment. Autologous B-LCLs were loaded with different amounts of the stimulating peptide 12-Cys (8–16) (concentrations between 10^{-6} and 10^{1} μM), and killing was detectable from 10^{1}–10^{-2} μM peptide concentration. The competitor peptide 12-Val (8–16) was added in excess amounts at 50 μM concentration. The effector-to-target ratio was 20/1 in this experiment. Cytolytic activity was blocked by the competitor peptide for all concentrations tested and the dose-response curve was moved two logs to the right

peptides were present during peptide pulsing together with β_2-microglobulin, and the target cells were washed before addition of the T cells. We found that at the stimulating peptide concentration of 0.01 μM specific lysis of CTL clone EG2.9 is blocked by the competitor peptide in excess amounts from about 50% specific lysis and down to zero (Fig. 3). When we repeated this experiment with the other homologous ras peptides as competitors (12-Gly, 12-Asp, and 12-Arg), we found comparable competition, meaning that all the peptides, including the wild-type peptide were able to bind to HLA-A3 (data not shown).

Furthermore, we wanted to investigate the level of peptide competition by using different concentrations of the competing peptide 12-Val (8–16) at a fixed stimulating peptide 12-Cys (8–16) concentration. The target autologous B-LCLs were pulsed with 0.01 μM of the stimulating peptide and the competitor peptide was titrated at different concentrations as indicated (Fig. 4). The 50% inhibition concentration for the competitor peptide 12-Val (8–16) was 0.2 μM.

Recognition of m-RNA transfected target cells expressing the 12-Cys mutation

For peptide-induced tumor-specific CTLs to have potentially clinical benefits the CTLs must also be able to recognize tumor cells and effect their specific killing. In this case we did not have any tumor cell lines that coexpressed both the HLA-A*0302 molecule together with the K-*RAS* mutation 12Gly→Cys, and we were not able to obtain autologous tumor samples from the patient. Therefore we used B-LCLs having the appropriate HLA-A*0302 molecule as target cells and transfected them with K-*RAS* m-RNA carrying the 12-Cys mutation. The control cells were treated in the same way but without 12-Cys mRNA before being examined in a Cr-release assay. CTL clone EG2.9 recognizes the target cells transfected with the 12-Cys mutation at comparable level with the peptide-pulsed target cells (Fig. 5). The transfected cells without 12-Cys mRNA was not

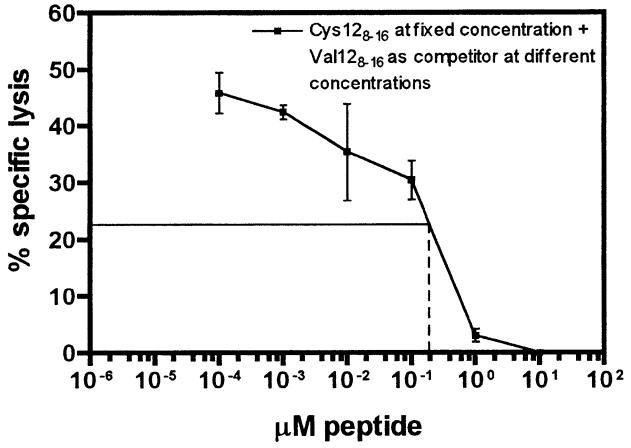

Fig. 4 Peptide competition experiment of CTL clone EG2.9. The level of peptide competition was examined using different concentrations of the competitor peptide 12-Val (8–16) at a fixed stimulating peptide concentration of 12-Cys (8–16). Autologous B-LCLs were used as target cells and loaded with 0.01 μM of the stimulating peptide and the competitor peptide was titrated at different concentrations as indicated. Effector-to-target ratio was 20/1 in this experiment. The 50% inhibition concentration for the competitor peptide 12-Val (8–16) was 0.2 μM

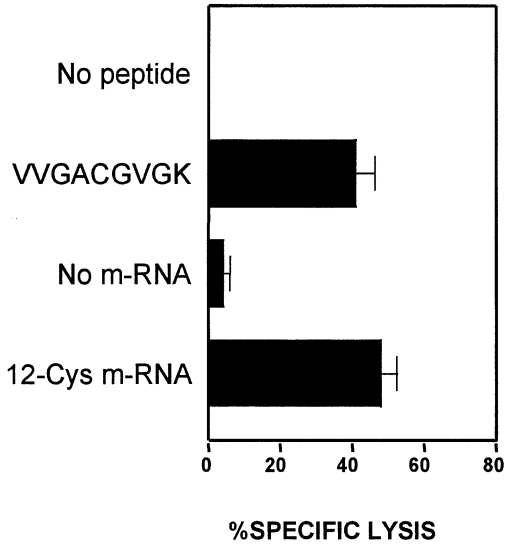

Fig. 5 Recognition of K-*RAS* 12-Cys transfected target cells by CTL clone EG2.9. To demonstrate recognition of endogenously processed mutant ras protein we created surrogate tumor cells by transfecting autologous B-LCLs with 12-Cys mRNA (concentration 5 μg/ml) using a mRNA-based electrotransfection technique [31]. Peptide-loaded (0.01 μM) B-LCLs and cells transfected without 12-Cys mRNA and cells in medium alone served as controls. Effector-to-target ratio for this experiment was 20/1. CTL clone EG2.9 demonstrated comparable recognition of both K-*RAS* 12-Cys transfected cells and peptide-loaded 12-Cys (8–16) target cells

recognized. This demonstrated that the peptide-induced CTL clone EG2.9 recognizes a processed form of the corresponding mutant ras protein, which is a prerequisite for such CD8+ CTLs to have a potential in vivo effector function.

Discussion

The development of effective vaccination protocols to immunize patients against their growing cancer is a major goal in human tumor immunology. As recently reported by our group, a vaccination protocol has been developed using intradermal injections of a mixture of mutant ras peptides in combination with GM-CSF as an adjuvant [24]. This vaccination protocol was used to immunize patients with adenocarcinoma of the pancreas, and immunity to mutant ras peptides was reproducibly induced in more than 50% of the patients treated.

Initially our emphasis focused on the impact of peptide vaccination on the development of the CD4+ T-cell response since the 17-mer peptides used for vaccination promiscuously bind to HLA class II molecules and are presented to CD4+ T cells in vivo. However, we also explored the hypothesis for the induction of CD8+ T-cell responses, which may have occurred as a result of further peptide processing in vivo, and which have been described earlier in our laboratory and elsewhere [18, 25, 26]. Parts of the ras molecule (around position 12 and nested within the vaccine peptide) have previously been shown to be presented by HLA-B35 [25] and HLA-A2 [26]. The identification of new peptide epitopes within antigens already known to be targets of antitumor CTLs may contribute to improved vaccines. The goal of the current study was to determine new HLA/peptide combinations that are recognized by CTLs and generated in vivo after vaccination. Furthermore, the assessment of whether such CTLs recognized endogenously processed tumor antigens was an important task of this investigation.

This work identified a nested epitope within the vaccine peptide used for immunization which carries the previously identified HLA-A*0302 binding motif [27]. The new peptide epitope was demonstrated to be presented by HLA-A*0302 and to a lower extent also by HLA-A*0301. The amino acid sequence of HLA-A*0302 and HLA-A*0301 differ only by three amino acids, and this may explain why HLA-A*0301 also presents the epitope (IMGT/HLA online database, http://www3.ebi.ac.uk/Services/imgt/hla).

In a previous evaluation of binding affinity of mutant ras peptides (T2-assay) Bertazzoli et al. [30] have described the binding of a sequence of the ras protein around codon 12 to HLA-A3. Our report, however, is the first documentation that T cells generated after peptide vaccination recognize the HLA-A3/ras-peptide complex. The peptide sequence (ras 8–16) evaluated by Bertazzoli et al. was identical with the newly characterized epitope described in the present study. Several (ras 8–16) peptide sequences carrying the more frequent mutations at codon 12, including the wild-type sequence, were able to bind [30]. In peptide-competition experiments with the CTL clone EG2.9 we confirmed that the homologous ras peptides (carrying the most common amino acid substitutions at position 12 or the wild-type sequence) are inhibitory and therefore capable of binding to the HLA-

A*0302 molecule. Together these experiments indicate that it should be possible to include these homologous ras epitopes in future cancer vaccines for use in HLA-A3$^+$ individuals.

The HLA-A3 genotype is present in about 20% of whites [31], which indicates that such mutant ras epitopes could be processed and presented on approximately 20% of all pancreatic adenocarcinomas. Taken together with earlier presented work this means that adenocarcinomas of the pancreas which are class I positive can function as target cells for such activated CTLs in patients positive for HLA-A2 [26], HLA-A3, and HLA-B35 [25]. These three HLA types (A2, A3, and B35) are present in at least 60% of the white population and are therefore frequently encountered among pancreas cancer patients.

In the present study peptide vaccination with GM-CSF of patient EG resulted in priming of a CD8$^+$ T-cell response. This most likely resulted from the intracellular processing by DCs in vivo, which take up the antigen and present it to T cells in the draining lymph node. The importance of DCs in immune responses to tumor antigens has been emphasized in view of emerging evidence for frequent defective antigen processing in tumor cells [32, 33]. DCs can internalize and process tumor antigens for efficient T-cell recognition of specific complexes between peptides and HLA class I or class II molecules expressed on their surface [12]. This ensures effective presentation of the antigenic peptide by cells that can deliver both the antigen-specific and the costimulatory signals, which are required for adequate T-cell activation. The present finding that CD8$^+$ T cell epitopes are generated in vivo after processing of vaccine components in DCs and subsequently are presented by HLA-A3 molecules to CD8$^+$ T cells extends upon previous work that has documented CD8$^+$ T-cell recognition of mutant ras after peptide immunization [25, 26]. To further strengthen this anti-ras response (generated by vaccination with 17-mer helper epitopes) both CD4 and CD8 peptide epitopes should be included in future peptide vaccine studies to induce more potent tumor rejection responses [34]. More potent immune responses may eventually provide a better clinical outcome for cancer patients.

The vaccination procedure described here, using mutant ras peptides and GM-CSF as an adjuvant, generates Th-1 responses in vivo. Importantly, the induced CD8$^+$ lymphocytes recognize processed mutant ras protein after transfection, which render these cells as functional effector cells. In contrast to this, Siegel et al. [35] found that immunization with a mutant ras peptide in complete Freund's adjuvant led to increased tumor growth in mice even though a CD4$^+$ T-cell response was induced. This surprising finding may be because the induced CD4$^+$ lymphocytes were unable to react to the intact mutant protein, or because these helper T cells were of a Th-2 type supporting antibody responses and not leading to cytotoxic T cell reactivity in situ.

Tumor-antigen specific T-cell responses following immunotherapy and particularly after administration of natural and synthetic anticancer vaccines have been studied in patients with cancer [36, 37]. Quantitation of antigen-reactive T cells before, during, and after therapy is crucial for future development of antitumor vaccines. Several methods have been developed to detect the frequency of antigen-specific T cells in the peripheral circulation of patients treated with anticancer vaccines [38]. These methods do not apply directly to our clinical trials since our main focus has been to induce T-helper responses by using promiscuous class II binding peptides as antigens in the vaccine. Accordingly, HLA class I typing has not been part of the inclusion criteria, and all relevant class I epitopes have not been defined. Therefore in the present study we used cloning by limiting dilution and thereafter performed cytotoxicity assays to assess CTL activity. The Cr-release assay provides the possibility of measuring lysis of tumor cells; however, the cytotoxicity assay is not acceptable for screening of CTL precursors in PBMC in clinical cancer vaccine trials. Other methods used to monitor antigen-reactive T cells before and after immunotherapy are cytokine-based assays, including intracellular staining of cytokines and quantification by flow cytometry or the Elispot assay, which is an antibody-based technique for quantitation of single cells secreting cytokines in response to antigen challenge. The Elispot assay is useful to monitor clinical protocols and offers an opportunity for quantitative assessments of CD8$^+$ as well as CD4$^+$ T-cell precursor frequencies in freshly isolated PBMC. The Tetramer-binding assay for direct assessment of ligand-binding T cells is another useful monitoring technique. By characterization of new peptide epitopes that bind to HLA molecules and stimulate CTL responses it is possible to include these epitopes in HLA-peptide complexes (tetramers) and use them for immune monitoring of vaccinated patients. Future clinical vaccine trials using new peptide/HLA tetramers may be able to achieve better methods for assessing/comparing immune response with clinical outcome, which is the final goal of such therapies.

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