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CYTOTOXIC CD4$^+$ AND CD8$^+$ T LYMPHOCYTES, GENERATED BY MUTANT p21-*ras* (12VAL) PEPTIDE VACCINATION OF A PATIENT, RECOGNIZE 12VAL-DEPENDENT NESTED EPITOPES PRESENT WITHIN THE VACCINE PEPTIDE AND KILL AUTOLOGOUS TUMOUR CELLS CARRYING THIS MUTATION

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Mutant p21-ras proteins contain sequences that distinguish them from normal ras, and represent unique epitopes for T-cell recognition of antigen-bearing tumour cells. Here, we examined the capacity of CD4$^+$ and CD8$^+$ T cells, generated simultaneously by mutant-ras-peptide vaccination of a pancreatic-adenocarcinoma patient, to recognize and lyse autologous tumour cells harbouring corresponding activated K-*ras* epitopes. The patient was vaccinated with a purified 17mer ras peptide (KLVVVGA*V*GVGKSALTI), containing the Gly12 → Val substitution. Responding T cells were cloned following peptide stimulation, and CD4$^+$ and CD8$^+$ peptide-specific cytotoxic T lymphocytes(CTL) were obtained. Transient pancreatic-adenocarcinoma cell lines(CPE) were established in cell culture from malignant ascites of the patient, and were shown to harbour the same K-*ras* mutation as found in the primary tumour. These cells were efficiently killed by the T-cell clones and CD8$^+$-mediated cytotoxicity was HLA-class-I-restricted, as demonstrated by inhibition of lysis by anti-class-I monoclonal antibodies. By employing as targets different class-I-matched tumour cell lines expressing a 12Val mutation, we were able to demonstrate HLA-B35 as the restriction molecule, and further use of peptide-sensitized EBV-B cells as target cells identified VVVGAVGVG as the nonamer peptide responsible for CD8$^+$-T-cell recognition. These data demonstrate that peptide vaccination with a single mutant p21-ras-derived peptide induces CD4$^+$ and CD8$^+$ CTL specific for nested epitopes, including the Gly → Val substitution at codon 12, and that both these T-cell sub-sets specifically recognize tumour cells harbouring the corresponding K-*ras* mutation. *Int. J. Cancer* 72:784–790, 1997.
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The *ras* p21 proto-oncogenes encode highly conserved intracellular proteins that are involved in cell proliferation and differentiation. ras proteins acquire their oncogenic potential as the result of single amino-acid substitutions restricted to codons 12, 13 or 61 (Bos, 1989). The presence of mutations at either of these positions result in proteins that are in an active GTP-bound state and contribute to the transformation process through constitutive transduction of growth-promoting signals (Seeburg *et al.,* 1984). Mutations in *ras* genes have been reported in a variety of human malignancies, and are detected in up to 90% of pancreatic adenocarcinomas (Almoguera *et al.,* 1988). Mutant p21 ras proteins are not expressed by normal cells and thus represent true cancer-specific proteins. Mutant-ras proteins or corresponding peptide sequences have been shown to be immunogenic in healthy individuals (Jung and Schluesener, 1991) and in cancer patients (Gedde-Dahl *et al.,* 1992*b*). Most responding T cells have been of the CD4$^+$ phenotype, but also human CD8$^+$ T cells isolated from a patient with adenocarcinoma of the colon, specific for a single *ras* mutation and capable of killing tumour cells harbouring the same mutation have been described (Fossum *et al.,* 1995). Thus, mutated p21 ras proteins may bear unique antigenic determinants for immune recognition by T cells, which play an important role in host anti-tumour activity (Greenberg, 1991).

T lymphocytes recognize antigens, through their αβ-T-cell receptor(TcR), as small peptides bound to major-histocompatibility-complex(MHC)-class-I or -class-II molecules displayed on the surfaces of antigen-presenting cells(APC) or target cells (Hunt *et al.,* 1992; Jardetzky *et al.,* 1991; Rudensky *et al.,* 1991; Townsend *et al.,* 1985). CD4$^+$ T cells recognize antigenic peptides (13–18 amino acids in length) presented in the context of class-II molecules (Hunt *et al.,* 1992; Rudensky *et al.,* 1991), whereas CD8$^+$ T cells recognize shorter peptides (8–10 amino acids in length) bound to the groove of class-I molecules (Jardetzky *et al.,* 1991). CD4$^+$- and CD8$^+$-T-cell sub-sets may be associated with cytotoxicity of tumour or virus-infected cells, and within the CD4$^+$ sub-set of T cells, Th1 cells are those responsible for lytic capacity (Erb *et al.,* 1990). Since activated p21 ras is intimately involved in the malignant phenotype of the cell and expresses antigens unique to cancer cells, such K-*ras* mutations may represent an attractive target for the induction of tumour-specific cytotoxic T lymphocytes(CTL). p21 ras is localized to the cytosol, where it is bound to the internal part of the plasma membrane (Willingham *et al.,* 1980) and is therefore potentially susceptible to the endogenous pathway of antigen processing and subsequent loading onto class-I or class-II molecules (Jardetzky *et al.,* 1991; Weiss and Bogen, 1991). Successful recognition of tumour cells harbouring point-mutated ras, would require presentation of endogenously processed antigenic epitopes bound to the appropriate MHC molecule on the target-cell surface in sufficient density for TcR recognition.

In a pilot clinical study, we vaccinated pancreatic-adenocarcinoma patients with synthetic ras-peptide-pulsed autologous APC, thereby eliciting peptide-specific T-cell responsiveness *in vivo* (Gjertsen *et al.,* 1995, 1996*a*). To characterize the peptide-specific CD4$^+$ T cells obtained by vaccination, T-lymphocyte clones(TLC) were generated from one of the vaccinated patients and studied in detail (Gjertsen *et al.,* 1996*c*). From these *in vivo*-activated proliferating T cells obtained after peptide immunization, we also obtained several CD8$^+$ TLC. In order to investigate whether these TLC were able to recognize and kill tumour cells from the patient, we generated transient tumour cell lines derived from ascites fluid harvested from the patient. We report here, that CD4$^+$ CTL and CD8$^+$ CTL produced by mutant-ras-peptide vaccination, demonstrated human-leukocyte-antigen(HLA)-restricted lysis *in vitro* of autologous tumour cells harbouring the same mutation. Our data indicate that overlapping peptide sequences contained within the 5-21, 12Val peptide used for vaccination are capable of activating CD4$^+$ and CD8$^+$ T cells *in vivo* simultaneously. Activation of CD4$^+$-proliferative as well as CD8$^+$-cytolytic responses to mutated p21 *ras* ensures an adequate immune response, since it is known from other systems that CD4$^+$ T-helper cells are required for maintaining and augmenting a CD8$^+$-T-cell response to an *in vivo*

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target (Walter *et al.*, 1995). Finally, identification of anti-ras-CD4+ and anti-ras-CD8+ responses activated simultaneously by a single mutant ras peptide *in vivo* demonstrate the potential of peptide vaccines for the development of cancer immunotherapy.

MATERIAL AND METHODS

Cell lines

The patient, 49 years old at diagnosis, had a moderately differentiated, unresectable adenocarcinoma of the pancreatic head. The patient's HLA type was HLA-A1,2; B8,35; DR3,6 (DRB1*0301, 1401); DQ1,2 (DQA1*0101,0501; DQB1*0201,0501.3). The K-*ras* mutation Gly12 → Val was identified in DNA from formalin-fixed paraffin-embedded tumour tissue using a highly sensitive technique modified from Kahn *et al.* (1991). The patient was vaccinated by 7 consecutive injections of autologous ras-peptide-pulsed APC, and a proliferative T-cell response in peripheral-blood mononuclear cells(PBMC) was induced (Gjertsen *et al.*, 1995). Transient pancreatic-adenocarcinoma cell lines(CPE) were established *in vitro* from malignant ascites fluid harvested from the patient. Among the adherent-cell population, CPE cells were positively selected by rosetting with the monoclonal antibody (MAb) Ber/EP4 (Latza *et al.*, 1990) coupled to Dynabeads (Dynal, Oslo, Norway) to separate them from the mixture of normal cells (*i.e.*, mesothelial cells and macrophages) contained within the malignant ascites. Autologous B-lymphoblastoid cell lines(B-LCL) were generated by Epstein-Barr-virus transformation of B cells from the patient. HLA-matched tumour cell lines harbouring the *ras* mutation Gly12 → Val were: SW 480 (ATCC, Rockville, MD) (HLA-A2, B8,17), FMEX (a gift from Dr. Ø. Fodstad, Oslo, Norway) (HLA-A2, B12, 35) and T24 (ATCC) (HLA-A1, 3, B18, 35). The NK target K562 (erythroleukaemia) was used as control in cytotoxicity assays. For generation of the pancreatic-adenocarcinoma cell lines, a large number of media and culture conditions were tested. Transient cultures of CPE cells could be established and were maintained in EGM-2 medium (Bio-Whittaker, Walkersville, MD) supplemented with 50 µg/ml gentamicin, 10% heat-inactivated FCS (GIBCO, Paisley, UK), 0.2 U/ml insulin, 10 ng/ml epidermal growth factor, 10 ng/ml insulin-like growth factor-1, 1 µg/ml heparin, 4 ng/ml fibroblast growth factor and 1 µg/ml hydrocortisone (all from Bio-Whittaker). All other cultures were grown in RPMI-1640 (Bio-Whittaker) supplemented with gentamicin, 15% heat-inactivated human pool serum (T cells) or 10% FCS (cell lines).

Peptides

Peptides encompassing residues 5-21 of p21 ras, KLVVVGAG-GVGKSALTI (single-letter code) or with a Gly-to-Val, -Arg, -Asp or -Cys substitution at residue 12, were synthesized and purified as described (Gedde-Dahl *et al.*, 1992*a*). Nonamers, covering the ras-peptide sequence from positions 4 to 20 and containing the 12Val substitution, were additionally synthesized (Table I). The peptides were dissolved in sterile water before filter sterilization, and were stored in aliquots at −70°C.

Antibodies

The following MAbs were used in blocking experiments and/or flow cytometry: W6/32, anti-HLA-class-I (hybridoma cells from the ATCC); B7/21, anti-HLA-DP (gift of Dr. F. Bach, Minneapolis, MN); SPV-L3, anti-HLA-DQ (gift of Dr. H. Spits, Palo Alto, CA); B8/11, anti-HLA-DR (gift of B. Malissen, Marseilles, France); L306.4, anti-LFA-3 (CD58) (Becton Dickinson, Mountain View, CA); Ber/EP4, pan-carcinoma (Dynal). Antibodies against ICAM were obtained through the Fifth International Workshop on Human Differentiation Antigens: MAY.029: anti-ICAM-1 (CD54). The PE-conjugated goat anti-mouse antibody (Southern Bio Technology, Birmingham, AL) was used as a secondary antibody in flow-cytometry analysis.

TABLE I – FINE MAPPING OF *ras* (12VAL)-SPECIFIC, HLA-B35-RESTRICTED TLC 69-30

Antigen	Peptide sequence[1]	Specific lysis (%)[2]
5-21 ras (12Gly)	KLVVVGAGGVGKSALTI	2
5-21 ras (12Val)	KLVVVGAV̄GVGKSALTI	6
12-20 ras (12Val)	V̄GVGKSALT	4
11-19 ras (12Val)	AV̄GVGKSAL	1
10-18 ras (12Val)	GAV̄GVGKSA	1
9-17 ras (12Val)	VGAV̄GVGKS	2
8-16 ras (12Val)	VVGAV̄GVGK	1
7-15 ras (12Val)	**VVVGAV̄GVG**	43
6-14 ras (12Val)	LVVVGAV̄GV	0
5-13 ras (12Val)	KLVVVGAV̄G	3
4-12 ras (12Val)	YKLVVVGAV̄	7
MART-1/Melan A	AAGĪGILTV	0

[1]Peptides were used to sensitize autologous EBV-B cells at a final concentration of 1 µM.–[2]A total of 5×10^4 ^{3}H-thymidine labelled, peptide-sensitized target cells were incubated with effector cells for 4 hr at an effector/target ratio of 5/1.

Generation of T-cell clones

Cloning of T cells was performed as described (Gjertsen *et al.*, 1996*c*). Briefly, responding PBMC from the patient were plated at 2×10^6 cells per well in 24-well plates (Costar, Cambridge, MA) and stimulated with the 12Val peptide at 25 µM. On day 9, cloning of T-cell blasts by limiting dilution was performed. T-cell blasts were counted in the microscope and seeded at 5 blasts per well onto Terasaki plates (Nunc, Roskilde, Denmark). As feeder cells, 2×10^4 autologous, irradiated (30 Gy) PBMC were used, and the cells were propagated with the 12Val peptide at 25 µM and 5 U/ml recombinant interleukin-2 (rIL-2) (Amersham, Aylesbury, UK). After 9 days, TLC were transferred onto flat-bottomed 96-well plates (Costar) with 1 µg/ml phytohaemagglutinin (PHA, Wellcome, Dartford, UK), 5 U/ml rIL-2 and allogeneic irradiated (30 Gy) PBMC (2×10^5) per well as feeder cells. After 6 days, TLC were transferred to 24-well plates with PHA/rIL-2 and 1×10^6 allogeneic, irradiated PBMC as feeder cells and screened for peptide specificity after 4 to 7 days.

Cytotoxicity assays

For ^{51}Cr-release cytotoxicity assays, target cells were incubated with 7.5 MBq ^{51}Cr and FCS in a total volume of 0.5 ml at 37°C for 1 hr. Target cells were washed 3 times, and seeded (2×10^3 cells per well) in 96-well U-bottomed microtiter plates (Costar). Effector cells were added at different numbers as indicated. Maximum and spontaneous ^{51}Cr release of target cells was measured after incubation with 5% Triton-X or medium respectively. Supernatants were harvested after 4-hr incubation at 37°C and radioactivity was measured in a Packard (Meriden, CT) Topcount microplate scintillation counter. Percentage of specific chromium release was calculated by the formula: (experimental release − spontaneous release)/(maximum release − spontaneous release) ×100. Spontaneous release was always below 20% of the maximum release.

For the JAM cytotoxicity assay (Matzinger, 1991), which measures intact DNA from living cells, target cells were labelled with 9.25 to 18.5×10^4 Bq/ml of ^{3}H-thymidine (Amersham) at 37°C in optimal growth conditions. The labelling time varied from 4 hr to overnight. Target cells were pelleted, washed once and seeded (5×10^3 cells per well) in 96-well U-bottomed microtiter plates (Costar). In peptide-pulsing assays, the different peptides were added to wells containing ^{3}H-thymidine-labelled autologous Epstein-Barr-virus-transformed(EBV-B) targets and incubated for 1 to 2 hr at 37°C. In some experiments, the EBV-B target cells were mild-acid-treated prior to peptide incubation to allow better peptide loading of target cells. T cells were added at different effector-to-target ratios. In inhibition experiments, ^{3}H-thymidine-labelled target cells (5×10^3 cells per well) 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. The plates were incubated for 4 hr at 37°C and then harvested before counting in a liquid-scintillation counter (Packard Topcount). Percentage of specific lysis was calculated by the formula: (spontaneous retained DNA − experimentally retained DNA)/spontaneous retained DNA × 100. The total incorporated counts (maximum) was measured in each assay to assure that spontaneous death did not exceed 10%.

Flow cytometry

Expression of HLA and adhesion molecules on cultured CPE cells were determined by flow-cytometry analysis. Non-activated and interferon-γ-(IFN-γ)activated CPE cells (cultured with 100 U/ml of (IFN-γ) for 3 days before analysis) were analyzed. Cells (5×10^4 per well) were treated with the appropriate antibody at a final concentration of 10 µg/ml for 30 min at 4°C, and washed twice before addition of the secondary PE-labelled antibody (Southern Bio Technology). After 30 min of incubation at 4°C, cells were washed twice and analysis was performed using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA).

*Identification of K-*ras *mutation*

DNA was extracted from various numbers of the established tumour cell line (CPE), and the DNA sequence was amplified with cloned Pyrococcus furiosus(pfu) polymerase and standard PCR conditions for 40 cycles at an annealing temperature of 53°C. The primers used for the amplification for exon 1 were (5KO-F) 5′ ATG ACT GAA TAT AAA CTT GTC 3′ and (3KO-GC) 5′ CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TCT ATT GTT GGA TCA TCA TAT TC 3′. The amplified fragments were run individually on a constant denaturant capillary electrophoresis(CDCE) as described by Kumar *et al.* (1995), and a mutant sequence was identified. DNA from a tumour cell line with a known K-*ras* mutation GGT → GTT at codon 12 (SW 480) was amplified and denatured together with the amplified fragment from CPE at 96°C for 5 min. Thereafter, the single-stranded DNA (SW 480 and CPE) was allowed to re-anneal at 65°C for 1 hr, then was run on CDCE in optimized conditions.

RESULTS

Establishment and characterization of pancreatic-adenocarcinoma cell lines

Initial experiments using the pan-carcinoma antibody Ber/EP4 demonstrated the presence of low numbers of cancer cells in the ascites fluid harvested from the patient in a late phase of the disease. On the basis of this observation, we tried to establish a tumour cell line in culture, since this would provide an invaluable tool for functional studies of the mutant-*ras*-specific T-cell clones generated after peptide vaccination. We performed a large number of experiments, including numerous variations of culture conditions and enrichment for tumour cells. In general, we were able to grow the tumour cells to some degree of purity and for some time, but eventually the growth rate declined or the cells were overgrown by more rapidly dividing cells. Finally, we were able to establish, with a high degree of reproducibility, transient cell lines of high purity, which we characterized by flow cytometry and K-*ras*-mutation analysis. The phenotypic characterization of cell-surface molecules expressed on cultured pancreatic-adenocarcinoma cell-s(CPE), with or without co-cultivation with IFN-γ, is shown in Figure 1. Cultured CPE cells express the epithelial antigen Ber/EP4, which is distinct from the contaminating cells, mainly constituted by mesothelial cells (Gjertsen *et al.*, 1996*b*) and peritoneal macrophages, which are non-epithelial and Ber/EP4-negative. The cell line expressed HLA-class-I molecules (W6/32), but was negative for HLA-DR molecules (B8/11) which could be up-regulated by IFN-γ stimulation. CPE cells display detectable levels of the adhesion molecules ICAM-1 and LFA-3, and the expression of ICAM-1 was further up-regulated by IFN-γ treatment.

To ensure that the CPE cell line, which is derived from metastatic cells, was representative of the primary tumour, we verified the presence of the original K-*ras* 12Gly → Val mutation (data not shown). To identify the K-*ras* mutation, amplification products from both CPE (showing a mutant sequence) and SW 480 (with a known 12Gly → Val mutation) were denatured together and allowed to re-anneal before being run on a CDCE (Kumar *et al.*, 1995). No additional peaks were identified when the mixed CPE/SW480 products were analyzed on CDCE, as compared with CPE alone. This procedure allowed the identification of a 12Gly → Val substitution, contained within the pancreatic-adenocarcinoma

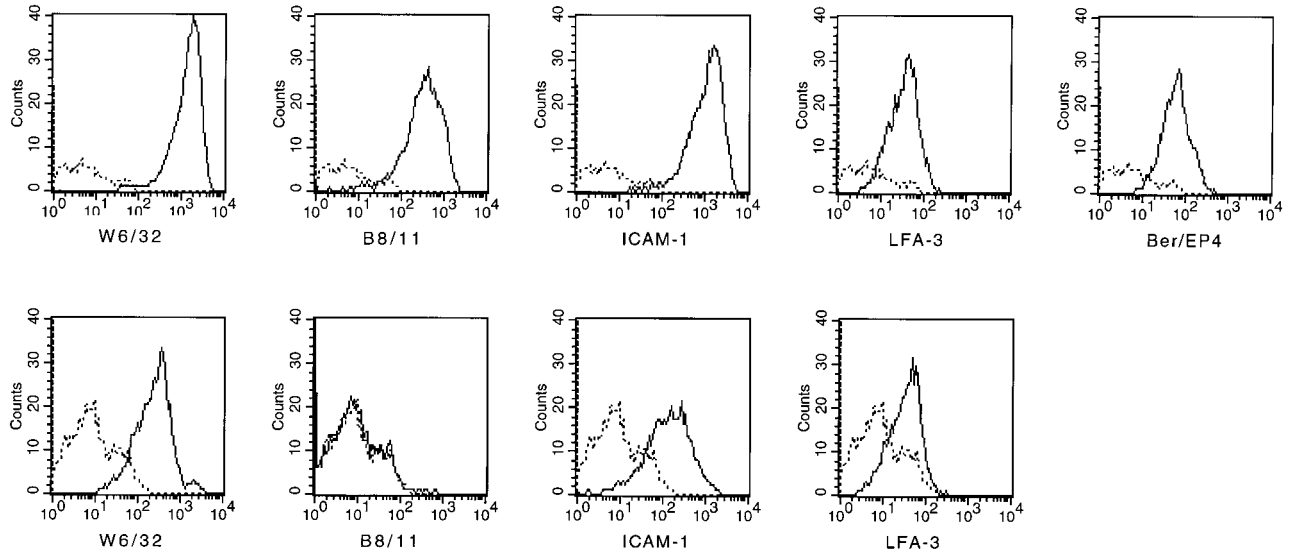

FIGURE 1 – Flow-cytometric analysis of CPE pancreatic-carcinoma cells. Cells were stained with MAbs directed against HLA-class-I (W6/32), HLA-DR (B8/11), ICAM-1, LFA-3 and Ber/EP4 molecules. Upper panel represents data obtained on CPE cells pre-treated with IFN-γ; lower panel demonstrates data obtained on tumour cells not pre-incubated with IFN-γ. Dashes represent background staining (cells treated with an irrelevant isotype-matched MAb plus the second antibody) and solid lines represent cells treated with the relevant MAb plus the second antibody.

cell line CPE, which is identical with the K-*ras* mutation originally identified in tumour tissue of the patient.

*Cytotoxic activity of CD4+ T-cell clones against autologous tumour cells bearing the K-*RAS *mutation Gly12 → Val*

After peptide vaccination, responding PBMC were cloned by limiting dilution, and several CD4+ and CD8+ TLC were obtained. The peptide-specific TLC 42-4, already described, proliferate specifically to the 12Val peptide using the HLA-DR6 molecule as the restriction element (Gjertsen *et al.,* 1996*c*). TLC 42-4 also exhibited cytolytic capacity against autologous EBV-B targets when pulsed with the mutated 12Val peptide, but not when pulsed with the control 12Gly peptide (Gjertsen *et al.,* 1996*c*). Cell-mediated cytotoxicity is an important pathway for elimination of antigen-bearing target cells, and the peptide-pulsed autologous EBV-B cell lines used in these experiments served as a surrogate for the actual autologous tumour cells at this stage. We therefore examined whether TLC 42-4 cells were also capable of lysing the autologous tumour cell line CPE. TLC 42-4 was tested against IFN-γ-treated CPE targets and autologous EBV-B cells either pulsed with the 12Val peptide or the 12Gly peptide, or alone. We also included the tumour cell lines T24, FMEX, SW 480 (12Val+, class 1+, class II−) and K562 cells as control targets. As shown in Figure 2, we observed specific lysis of CPE cells expressing the 12Val mutation at all effector-to-target ratios tested. With control EBV-B targets, lytic activity was observed only in the presence of exogenously added 12Val peptide, but not in the presence of 12Gly peptide or in EBV-B cells alone. The NK target K562 and the class-II-negative tumour cell targets (T24, FMEX, SW 480) were not lysed. Additionally, specific lysis of CPE targets was blocked by an anti-HLA-DR MAb (B8/11) (Fig. 3) and not by anti-class-I or anti-DQ or -DP MAbs; this result is consistent with our reported finding of HLA-DR6 as the 12Val-peptide-presenting molecule for TLC 42-4 (Gjertsen *et al.,* 1996*c*). Taken together, these data demonstrate that mutant *ras* in these tumour cells can be processed and presented in the context of HLA-DR6 following IFN-γ treatment.

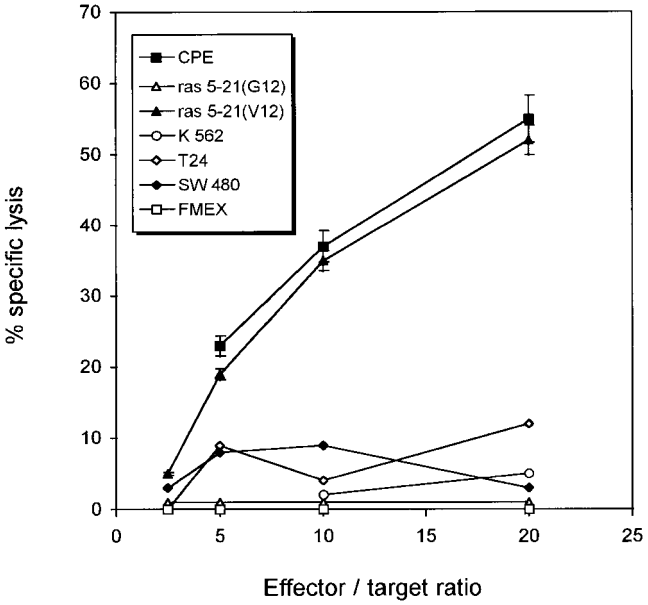

FIGURE 2 – Cytotoxicity against K-*ras* 12Gly → Val expressing pancreatic-carcinoma cells (CPE) by TLC 42-4. Cytotoxic activity was also examined against autologous EBV-B cells pulsed either with the *ras* 5-21 (V12)-peptide or with the *ras* 5-21 (G12) peptide at a final concentration of 15 μM. The tumour target cells FMEX, SW 480 and T24 (class I+, class II−) as well as the NK-target K562 were used as controls. Data represent percent specific lysis against ^{3}H-thymidine-labelled targets in a 4-hr assay and are expressed as the mean ± SD of triplicate cultures.

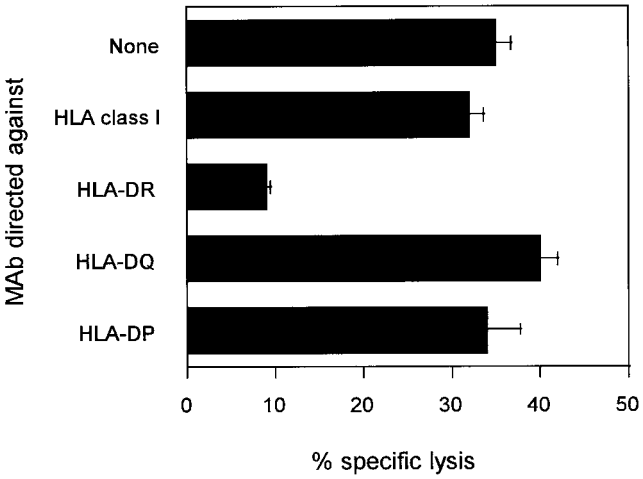

FIGURE 3 – Inhibition of anti-*ras* CTL activity of TLC 42-4 by anti-HLA-DR antibodies. The functional role of CTL activity against the autologous tumour cell line (CPE) was evaluated using MAbs directed against HLA-class-I (W6/32), HLA-DR (B8/11), HLA-DQ (SPV-L3) and HLA-DP (B7/21) molecules at a final concentration of 10 μg/ml. Results obtained with an effector/target ratio of 10/1 are shown. Data represent percent specific lysis against ^{3}H-thymidine-labelled CPE targets and the various MAbs in a 4-hr assay, with activity expressed as the mean ± SD of triplicate cultures.

Cytotoxic activity of CD8+ T-cell clones against autologous tumour cells and demonstration of HLA-class-I-restriction requirements

CD8+ CTL were obtained after cloning of T-cell blasts present in PBMC from a pancreatic-carcinoma patient after mutant-ras-peptide vaccination (Gjertsen *et al.,* 1996*c*). TLC 69-30 were selected for further characterization and express the cell-surface phenotype CD3, CD8 and TcR αβ (data not shown). TLC 69-30 exhibit lysis of autologous tumour cell targets when tested at different effector-to-target ratios (Fig. 4), indicating that they are directed against a tumour-derived antigen, such as mutant ras. Control cells, *i.e.,* mesothelial cells derived from the same ascitic fluid, were not killed (data not shown). In order to verify that the antigen recognized is associated with mutant *ras,* and to identify the HLA-class-I molecule presenting the putative mutant-ras peptide to the CTL, different 12Val expressing tumour cell lines carrying one or more HLA-class-I molecules in common with those of the patient were used as targets. TLC 69-30 demonstrated lysis of the bladder-carcinoma cell line T24 (12Val+, HLA-Al+, B35+) and the melanoma cell line FMEX (12Val+, HLA-A2+, B35+), but not of the colon-carcinoma cell line SW 480 (12Val+, HLA-A2+, B8+) (Fig. 4). The autologous EBV-B cells (12Val−, HLA-A1+, A2+, B8+, B35+) were not lysed. Killing was not due to NK activity of the CTL, since the natural killer target K562 was not lysed. These results suggest that TLC 69-30 recognize an endogenously processed 12Val epitope in the context of HLA-B35. That specific lysis of CPE cells was HLA-class-I-restricted was further supported by experiments involving MAbs directed against HLA-class-I and -class-II antigens. The results, illustrated in Figure 5, demonstrate that the cytolytic effect of TLC 69-30 on CPE targets could be blocked by a pan-reactive HLA-class-I MAb (W6/32), but remained unaltered in the presence of MAbs directed against HLA-class-II DR, DQ and DP antigens. Taken together with the results obtained with the different 12Val-expressing tumour cell lines, these data demonstrate HLA-class-I restriction and indicate that HLA-B35 is the restricting molecule of TLC 69-30.

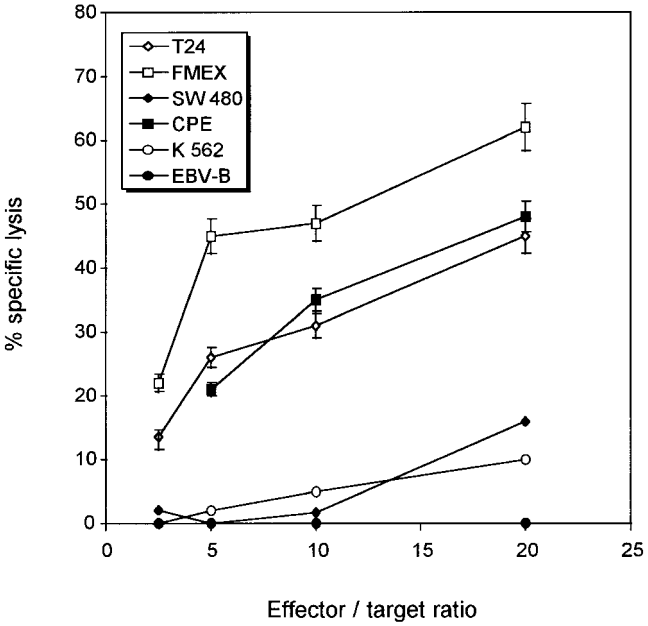

FIGURE 4 – Lysis of autologous tumour cells and different HLA-B35$^+$ tumour cell lines by TLC 69-30. Targets were T24 (HLA-A1, B35, Val 12$^+$); FMEX (HLA-A2, B35, Val 12$^+$); SW 480 (HLA-A2, B8, Val 12$^+$); CPE (autologous pancreatic-adenocarcinoma cells, HLA-A1, 2, B8, 35, Val 12$^+$); K562 and EBV-B (autologous EBV-B cells, HLA-A1, 2, B8, 35, Val 12$^-$). Data represent percent specific lysis against ^{3}H-thymidine-labelled target cells in a 4-hr assay at different effector/target ratios. Values are expressed as the mean of triplicate cultures ± SD.

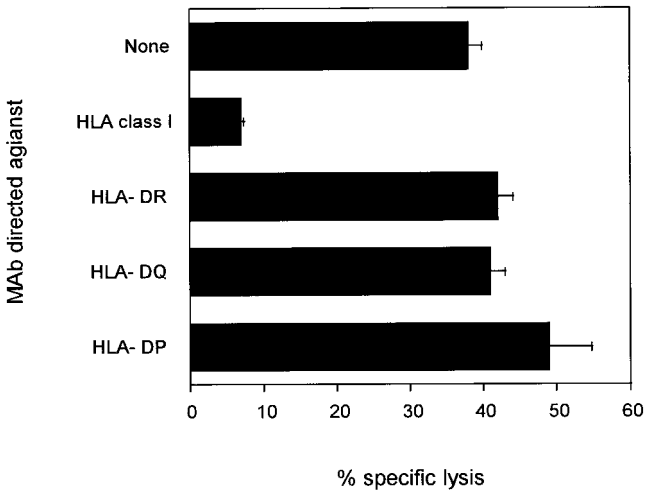

FIGURE 5 – Inhibition of anti-ras CTL activity of TLC 69-30 by anti-HLA-class-I antibodies. The functional role of CTL activity against the autologous tumour cell line (CPE) was evaluated using MAbs directed against HLA-class-I and -class-II molecules at a final concentration of 10 µg/ml. Results obtained with an effector/target ratio of 10/1 are shown. Data represent percent specific lysis against ^{3}H-thymidine-labelled CPE targets and the various MAbs in a 4-hr assay, with activity expressed as the mean ± SD of triplicate cultures.

Identification of the peptide recognized by the HLA-B35-restricted TLC 69-30

To identify the mutant-ras peptide recognized by TLC 69-30, a panel of nonamer peptides (Table I) spanning positions 4 to 20 of p21 ras containing the Gly → Val substitution at position 12 were synthesized. Acid-stripped autologous EBV-B cells were pulsed with these peptides and control peptides, as indicated in Table I. Only the peptide 7-15 ras (12Val) was capable of stimulating CTL activity (Table I). The specificity of CTL recognition for the appropriate mutant peptide was illustrated by the absence of lysis observed with the peptide expressing normal ras sequence. In addition, an irrelevant melanoma-associated peptide, MART-1/Melan-A peptide, was incapable of stimulating CTL activity (Table I). The sensitivity of TLC 69-30 to exogenous peptide was examined in a dose-response experiment using peptide-sensitized EBV-B cells as target cells (Fig. 6). Anti-*ras* CTL activity was detectable over a several-log range, with maximal lysis at 1×10^{-6} M and half maximal response at 1×10^{-9} M peptide concentration.

DISCUSSION

The purpose of vaccination of cancer patients is to selectively expand T-cell precursors specific for tumour antigens, with the hope that these cells will home to the tumour and exert anti-tumour activity *in vivo*. In the present report, we provide data generated by analysis of the immune response against mutant *ras* that occurred following vaccination of a patient with a synthetic ras peptide corresponding to the mutation found in his tumour. We have already reported that vaccination resulted in the generation of a specific immune response that could be detected in peripheral blood sampled around day 40 after the start of vaccination (Gjertsen *et al.*, 1995, 1996*a*), after which the responding cells disappeared from the blood. In the patient, the immune response was associated with prolonged life and massive T-cell infiltration into the tumour (Gjertsen *et al.*, 1995, 1996*a*). Here we demonstrate the functional importance of responding cells by showing that they are capable of killing tumour cells derived from the same patient. CTL responses against mutated-ras-derived epitopes can thus be induced *in vivo* in pancreatic-carcinoma patients after ras-peptide vaccination. This indicates the presence in the T-cell repertoire of pancreatic-carcinoma patients, of mutant-*ras*-specific precursor CTL which can be activated by appropriate immunisa-

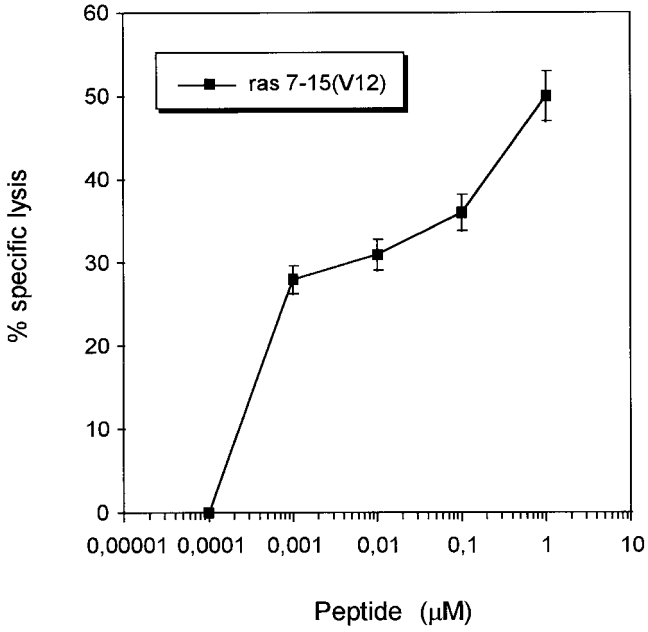

FIGURE 6 – Peptide sensitivity for lysis by TLC 69-30. The CD8$^+$ CTL clone was tested for sensitivity to peptide for lysis of ^{3}H-thymidine-labelled EBV-B targets in 4-hr dose-response experiments at an effector/target ratio of 10/1. Lysis in the absence of peptide or in the presence of the Melan-A/Mart-1 peptide was <1%. Data are expressed as the mean ± SD of triplicate cultures.

tion. We have earlier described different CD4+ TLC, from the same patient, which recognize the immunizing peptide in the context of HLA-DR6 or DQ2 (Gjertsen *et al.*, 1996*b*); we have now extended our findings to include CD8+ TLC that recognize cancer cells carrying the same *ras* mutation, and are specific for a ras peptide presented by HLA-B35.

Crucial for the initiation of effective anti-tumour immune responses *in vivo* is the presentation of antigenic peptides in an immunogenic context. Vaccination of patients with large amounts of peptide-pulsed autologous APC (containing a small fraction of dendritic cells) may stimulate T-cell recognition of the peptide antigen within regional lymphoid organs. Dendritic cells can efficiently stimulate T-cell recognition of specific complexes between peptides and HLA-class-I or -class-II molecules expressed on their surface (Steinman, 1991). This ensures effective presentation of the antigenic peptide by cells that can deliver both the antigen-specific and the co-stimulatory signals required for adequate T-cell activation (Schwartz, 1990). Of particular importance is concomitant activation of the T-helper and the effector arms of the immune system. In viral antigen systems, with influenza-nucleoprotein-derived peptides, both CD4+ and CD8+ epitopes have been shown to exist as overlapping or nested peptide sequences, which may have important physiologic implications for the generation of a more efficient host anti-pathogen cellular immune response (Carreno *et al.*, 1992). Our earlier report demonstrating CD4+ and CD8+ T cells specific for a 13Gly → Asp mutation (Fossum *et al.*, 1994), and the data presented here, as well as results obtained from a murine system (Abrams *et al.*, 1996), demonstrate that this is also the case for mutant *ras*. Although the main focus in tumour immunology of late has been on the identification of tumour-specific peptides recognized by CD8+ CTL, it is clearly important to identify peptide sequences capable of eliciting CD4+-T-cell recognition, thereby generating help for CD8+ T cells possibly capable of recognizing native antigens endogenously presented by the cancer cells. These cells are also an important component of a functional immunological memory.

Tumour regression may be considered the ultimate endpoint in evaluation of an immune response to cancer peptide vaccines. The assessment of tumour regression, however, needs specially designed studies allowing careful tumour evaluation. MHC-class-I or -class-II-restricted CTL are probably crucial elements of the cellular tumour defence. Therefore, determination of anti-ras CTL *in vivo* may serve as the operational endpoint guiding the development of effective tumour vaccines directed against mutant *ras*. We provide evidence that CD4+, HLA-DR6-restricted T cells, activated *in vivo* through peptide vaccination, are capable of recognizing and lysing autologous tumour cells *in vitro*. The tumour cells in this case require IFN-γ stimulation to express HLA-class-II molecules on the surface (HLA-DR). Classically, endogenous antigens are processed and presented on the cell surface bound to HLA-class-I molecules for presentation to CD8+ T cells. However, numerous endogenous antigens have now been shown to have access to the HLA-class-II pathway. This report demonstrates that tumour cells expressing unmanipulated levels of the p21-ras protein have the capacity to be presented in the context of HLA-class-II molecules for recognition by CD4+ T cells. In a different system involving EBV-transformed B-cell lines transduced with the *ras* oncogene containing a 12Val mutation, efficient lysis was observed with *in vitro*-generated CD4+ T-cell lines as effector cells (Tsang *et al.*, 1994), further supporting the notion that mutant-ras epitopes may be presented in the context of relevant HLA-class-II molecules following endogenous processing. Together these data provide strong evidence that such cells, in addition to their helper function, may contribute as effector cells *in vivo*. However, whether these cells are capable of lysing tumour cells *in vivo*, where expression of MHC-class-II molecules requires induction by cytokines such as INF-γ and TNFα, remains to be determined. It is, however, conceivable that, following recognition of ras peptides *in situ* in the context of infiltrating professional APC, sufficient amounts of cytokines may be produced.

Furthermore, we demonstrate that a CD8+-CTL clone, activated simultaneously by peptide vaccination, will kill the autologous pancreatic cancer cells. The CPE cells express HLA-class-I molecules constitutively, and CD8+ recognition and efficient lysis was evident without IFN-γ treatment, which indicates that the T cells may also effectively lyse tumour cells *in vivo*. This is in contrast to the report of a CD8+-CTL clone isolated from a colon-carcinoma patient and specific for a 13Gly → Asp mutation, which was shown to lyse HLA-matched colon-carcinoma cells expressing the 13Gly → Asp mutation only after IFN-γ treatment (Fossum *et al.*, 1995). Moreover, by using a panel of overlapping peptides, we identify the 12Val peptide actually being recognized by the CD8+ CTL in the context of HLA-B35 as a 9-mer peptide sequence of mutant K-*ras*. This 9-mer sequence was originally contained within the 17-mer peptide used for vaccination, and further supports the assumption of overlapping T-cell epitopes in nearby co-existence around position 12 of p21 ras.

In conclusion, our findings demonstrate that point-mutated *ras*-oncogene products present in cancer cells expressing relevant HLA-class-I and -class-II molecules can serve as targets for T-cell-mediated cytotoxicity, suggesting sufficient TcR affinity for recognition of endogenously derived epitopes. Moreover, immunotherapy protocols employing mutant-ras peptides may elicit such T cells *in vivo* following vaccination of cancer patients. At present, the challenge is to develop more potent peptide immunization, in order to enhance the anti-ras T-cell response to a level ensuring efficient tumour rejection *in vivo*.

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