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INTRADERMAL RAS PEPTIDE VACCINATION WITH GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR AS ADJUVANT: CLINICAL AND IMMUNOLOGICAL RESPONSES IN PATIENTS WITH PANCREATIC ADENOCARCINOMA

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***K-RAS* mutations are frequently found in adenocarcinomas of the pancreas, and induction of immunity against mutant ras can therefore be of possible clinical benefit in patients with pancreatic cancer. We present data from a clinical phase I/II trial involving patients with adenocarcinoma of the pancreas vaccinated by i.d. injection of synthetic mutant ras peptides in combination with granulocyte-macrophage colony-stimulating factor. Forty-eight patients (10 surgically resected and 38 with advanced disease) were treated on an outpatient basis. Peptide-specific immunity was induced in 25 of 43 (58%) evaluable patients, indicating that the protocol used is very potent and capable of eliciting immune responses even in patients with end-stage disease. Patients followed-up for longer periods showed evidence of induction of long-lived immunological memory against the ras mutations. $CD4^+$ T cells reactive with an Arg12 mutation also present in the tumor could be isolated from a tumor biopsy, demonstrating that activated, ras-specific T cells were able to selectively accumulate in the tumor. Vaccination was well tolerated in all patients. Patients with advanced cancer demonstrating an immune response to the peptide vaccine showed prolonged survival from the start of treatment compared to non-responders (median survival 148 days *vs.* 61 days, respectively; $p = 0.0002$). Although a limited number of patients were included in our study, the association between prolonged survival and an immune response against the vaccine suggests that a clinical benefit of ras peptide vaccination may be obtained for this group of patients.**

Key words: *peptide vaccination; pancreatic cancer; mutant ras; granulocyte-macrophage colony-stimulating factor; adenocarcinoma*

Most patients with pancreatic adenocarcinoma are inoperable at the time of diagnosis. There is currently no effective non-surgical treatment option, and median survival time remains short (3 to 4 months). The small subgroup of operable cases (approx. 10%) has a median survival time of approximately 18 months.[1] Advances in treatment options for patients with pancreatic cancer are urgently needed.

Mutations in the proto-oncogenes of the RAS family are frequent in human malignancies, and *K-RAS* mutations are found in most adenocarcinomas of the pancreas.[2] In addition, the fact that most RAS mutations are confined to codons 12, 13 and 61[3] makes the protein an attractive target for induction of tumor-specific T cells. Previous studies have shown that immune responses to mutated ras peptides and proteins can occur spontaneously in patients with malignancy or can be elicited in normal individuals.[4–10] Peptide-specific T-cell responsiveness against mutant ras can also be induced *in vivo* in cancer patients by vaccination with antigen-presenting cells (APCs) loaded *ex vivo* with ras peptides or ras peptides emulsified in adjuvant.[11,12] These peptides, which are 13 to 17 amino acids in length, represent natural ras epitopes[13–15] and are designed primarily to induce CD4 T helper-specific immune responses. The ras-specific effector cells involved are generally of the $CD4^+$ phenotype, but $CD8^+$ T cells specific for nested epitopes encompassing the ras mutation have also been described.[14] Both T-cell subsets can lyse autologous tumor cells or HLA-matched cancer cell lines expressing the corresponding *K-RAS* mutation,[13–15] demonstrating that relevant peptide epitopes are generated by endogenous processing of mutant p21 ras in tumor cells. Together these findings demonstrate that the T-cell repertoire in both healthy individuals and cancer patients contains T cells capable of recognizing mutant ras and that these T cells can be selectively expanded in cancer patients after vaccination. The low frequency of spontaneous T-cell response against mutant ras in patients having tumors with *K-RAS* mutations (this report and unpublished data) indicate that mutant ras is poorly immunogenic in cancer cells. This contrasts with the immunogenicity of mutant ras peptides *in vivo,* which can be demonstrated by vaccination.

To initiate a clinically relevant immune response against mutant ras in cancer patients, *i.e.,* to overcome the state of non-responsiveness toward the tumor *in vivo,* the peptide antigens must be delivered in an immunogenic context. Dendritic cells (DCs) are APCs specialized for the induction of a primary T-cell response[16,17] and can induce anti-tumor immunity *in vivo.*[18–20] Purified peptide epitopes given i.d. in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) has been reported to induce efficient T-cell responses against peptide antigens in experimental animals[21] and to enhance T-cell responsiveness to melanoma-associated peptides in melanoma patients and patients with breast and ovarian carcinomas.[22,23] The adjuvant effect of the cytokine GM-CSF is related to the maturation and activation of DCs, which after antigen uptake will move to an adjacent lymph node and activate effector T cells.[16,17,24]

To induce anti-ras immunity in patients with pancreatic cancer, we developed a vaccination protocol based on i.d. injection of mutant ras peptides in combination with GM-CSF. Here, we report

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the successful induction of T-cell immunity associated with clinical responses after i.d. vaccination using mutant ras peptides in combination with GM-CSF in patients with pancreatic cancer. Our purpose was 3-fold: *(i)* to assess the safety and toxicity of this treatment, *(ii)* to determine the response rate of mutant ras peptide vaccination in terms of immunological response and *(iii)* to determine the tumor response (development of metastasis or recurrence of primary tumor in resected patients) or survival time (patients with advanced disease). The reason for including both patients with resectable tumor and patients with advanced disease was to assess whether the tumor burden would have an impact on the rate of immunological responses.

MATERIAL AND METHODS

Patient selection

Patients were recruited into 2 clinical studies, 1 including potentially curatively resected patients with pancreatic adenocarcinoma (CTN RAS 95002) and the second consisting of patients with advanced disease (CTN RAS 97004). Treatment protocols were approved by the Regional Ethics Committee Health Regions I and II, and studies were performed according to the principles of the Helsinki declaration. Forty-eight patients were enrolled, and clinicopathological variables and response data are summarized in Tables I and II. Inclusion required histologically proven adenocarcinoma of the pancreas (either resectable or advanced) and a life expectancy of at least 8 weeks. Patients with active infection with hepatitis virus or HIV and patients treated with chemotherapy or radiation therapy within 4 weeks prior to vaccination were excluded. All patients gave informed consent before being enrolled. Recruitment started in November 1996, and the study was closed in November 1998.

DNA extraction and K-RAS mutation analysis

DNA was extracted from formalin-fixed, paraffin-embedded tumor material. Paraffin was removed from 5×10 μm sections by repeated xylene treatments, and ethanol-dried pellets were digested with proteinase-K followed by phenol-chloroform extraction and DNA precipitation. Enriched PCR was used to obtain mutant DNA template.[25] If a mutant product was revealed by restriction analysis, the specific mutation was identified by direct sequencing using an Applied Biosystems (Rotterdam, the Netherlands) 373 DNA sequencer.

Peptides

Synthetic ras peptides encompassing residues 5–21 of p21 ras were synthesized and purified as clinical grade reagents under GMP conditions (Norsk Hydro, Porsgrunn, Norway). The normal sequence of ras p21 reflecting positions 5–21 is Lys-Leu-Val-Val-Val-Gly-Ala-Gly-Gly-Val-Gly-Lys-Ser-Ala-Leu-Thr-Ile (KLVVVGAGGVGKSALTI). The mutant ras 17-mer peptides used as vaccines reflected the substitution of Gly at position 12 with an Asp, Cys, Val or Arg residue. Each of the peptides was supplied as a freeze-dried, sterile white powder soluble in water. Nonamer peptides covering residues 4–20 of p21 ras and containing the Val12, Arg12, Asp12 or Cys12 substitution were additionally synthesized and used for determination of CTL epitopes *in vitro.*

Vaccination protocol

One week before vaccination, the following baseline studies were performed: physical examination (including medical history); hematological testing for hemoglobin, hematocrit, C-reactive protein, leukocytes and platelet count; blood chemistry panel; and assessment of performance status. CT scans of the abdomen were obtained within 8 weeks prior to inclusion. Blood was also taken for testing of general immunocompetence and pre-vaccination T-cell reactivity against the vaccine peptides.

Eligible patients received 4 vaccinations at weekly intervals into the right para-umbilical area and a booster vaccination at weeks 6 and 10. Briefly, weekly i.d. injections of 100 μg (1 mg/ml) of a single mutant ras peptide corresponding to the *K-RAS* mutation identified (resectable patients) or a mixture of 4 mutant ras peptides (final concentration of each peptide 1 mg/ml, non-resectable patients) in 0.1 ml saline were given. This protocol was chosen since valuable time was lost in the process of determining the *K-RAS* mutation. The 4 peptides corresponded to the most frequent *K-RAS* mutations found in pancreatic adenocarcinoma.[3] Fifteen minutes prior to peptide injections, 40 μg recombinant human GM-CSF (Leucomax; Schering-Plough, Cork, Ireland) in 0.1 ml saline were administered by i.d. injection. Comprehensive immunological testing, assessment of adverse drug reactions, blood screening, physical examination and assessment of performance status were done at each vaccination visit. At the end of the study, a complete clinical and immunological screening identical to the initial work-up was performed. A follow-up clinical protocol was designed to include patients who demonstrated an immune response to the vaccine and had a stable clinical situation following completion of the protocol (after week 14). The follow-up protocol started 3 to 6 months after the first protocol, and the interval between additional booster vaccinations was 3 to 4 months for up to 2 years.

Delayed-type hypersensitivity

Delayed-type hypersensitivity (DTH) skin tests were performed with either a single peptide or a peptide mixture at each vaccination. The single peptide (100 μg) or the mixture of peptides (100 μg of each of the 4 mutant peptides) dissolved in saline were injected i.d. (without GM-CSF) into the left para-umbilical area at a site distant from the vaccination site. A positive skin test reaction was defined as 5 mm diameter erythema and induration 48 hr after i.d. injection. The patient was instructed to measure the diameter of the erythema/induration and report it to the clinician, who recorded the skin test as positive or negative in the clinical report form.

TABLE I – PATIENT CHARACTERISTICS AND RESPONSE TO PEPTIDE VACCINATION IN SURGICALLY RESECTED PANCREATIC CANCER

Patient	*K-RAS*	Tumor differentiation	Immunological response		Tumor response
			DTH	T-cell reactivity (SI)[1]	
1 (F/73)	Val12	Unknown	+	5.5	NED[2] at 31 months
2 (M/35)	Cys12	Poor	+	3.2	NED at 39 months
3 (M/61)	Val12	Well	−	1.0	Lung metastases 12 months
4 (M/68)	Asp12	Well	−	1.0	Local recurrence 20 months
5 (F/61)	Arg12	Moderate	−	3.4	Lung metastases 9 months
6 (M/59)	Arg12	Moderate	−	1.0	NED at 30 months
7 (M/72)[3]	Asp12	Poor	NT[4]	NT	Liver metastases 1 month
8 (M/64)	Val12	Well	−	1.0	NED at 22 months
9 (F/58)	Val12	Unknown	+	8.3	Local recurrence 10 months
10 (F/59)	Asp12	Moderate	+	4.5	Lung metastases 7 months

[1]T-cell reactivity was evaluated in post-vaccination PBMC expressed as stimulatory index (SI). There was no T-cell reactivity in pre-vaccination samples in any of the patients.–[2]NED, no evidence of disease.–[3]Patient withdrawn after 1 month due to liver metastases, excluded from efficacy analysis.–[4]NT, not tested.

TABLE II – PATIENT CHARACTERISTICS AND RESPONSE TO PEPTIDE VACCIATION IN ADVANCED DISEASE

Patient	*K-RAS*	Tumor differentiation	Immunological response		Tumor response	
			DTH	T-cell reactivity (SI)[1]	Clinical response[2]	Survival time (days)[3]
11 (F/51)	Asp12	Moderate	−	1.0	PD	36
12 (F/56)	WT[7]	Unknown	+	1.0	PD	129
13 (F/63)	Arg12	Well	+	6.2	PR 28 months	940
14 (F/74)	Cys12	Moderate	−	1.0	PD	32
15 (F/67)	Asp12	Moderate	−	1.0	PD	37
16 (M/60)	WT	Moderate	−	1.0	PD	99
17 (F/68)	Val12	Unknown	+	7.7	SD 6 months	209
18 (M/69)	Val12	Unknown	+	1.0	PD	68
19 (F/62)	Asp12	Poor	+	1.0	PD	80
20 (F/54)[5]	Cys12	Unknown	NT[6]	NT	PD	19
21 (M/59)	Arg12	Poor	+	3.1	PD	54
22 (F/77)	Arg12	Unknown	−	1.0	PD	56
23 (M/54)	Asp12	Well	−	1.0	PD	30
24 (F/56)	NT	Unknown	+	1.0	PD	78
25 (M/61)	NT	Unknown	−	1.0	PD	79
26 (F/57)	NT	Unknown	−	1.0	PD	66
27 (M/74)	NT	Unknown	−	1.0	PD	104
28 (F/78)	NT	Unknown	−	5.0	SD 3 months	82[4]
29 (F/82)	NT	Moderate	+	38.6	SD 23 months	785
30 (F/48)	NT	Unknown	+	4.6	PD	145
31 (F/77)	NT	Unknown	−	1.0	PD	120
32 (F/59)	WT	Moderate	+	1.0	SD 6 months	221
33 (F/60)[5]	NT	Unknown	NT	NT	PD	21
34 (F/66)[5]	NT	Unknown	NT	NT	PD	10
35 (M/65)	NT	Unknown	+	3.5	SD 7 months	231
36 (F/74)	NT	Unknown	+	9.6	SD 4 months	151
37 (F/65)	NT	Moderate	+	1.0	PD	103
38 (F/68)	NT	Unknown	−	1.0	PD	105
39 (F/51)	NT	Unknown	−	1.0	PD	48
40 (M/66)	NT	Unknown	+	1.0	SD 8 months	289
41 (F/62)	NT	Unknown	−	2.0	PD	52
42 (M/63)	NT	Unknown	−	7.8	PD	56
43 (M/47)	Val12	Unknown	+	1.0	SD 11 months	372
44 (M/71)	Arg12	Poor	+	10.3	SD 6 months	195
45 (M/66)[5]	NT	Unknown	NT	NT	PD	21
46 (F/53)	Val12	Unknown	+	32.6	SD 10 months	347
47 (M/69)	NT	Poor	−	1.0	PD	46
48 (M/75)	NT	Moderate	−	1.0	PD	108

[1]T-cell reactivity was evaluated in post-vaccination PBMCs expressed as stimulatory index (SI). There was no T-cell reactivity in pre-vaccination samples in any of the patients.–[2]Clinical response: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.–[3]Survival time: days from first vaccination until death.–[4]Patient died of apoplexia.–[5]Patient excluded from efficacy analysis.–[6]NT, not tested.–[7]WT, wild-type.

Monitoring of anti-ras T-cell responses

Prior to vaccination and at each visit, 10 to 20 ml ACD blood were drawn, to assess proliferative T-cell responses. Peripheral blood mononuclear cells (PBMCs) were prepared from peripheral blood using density centrifugation over Ficoll-Hypaque (Lymphoprep; Nycomed, Oslo, Norway) and seeded 10^5/well in round-bottomed 96-well plates (Costar, Cambridge, MA) in 100 μl X-VIVO 10 medium (Biowhittaker, Walkersville, MD) supplemented with the immunizing peptide at 25 μM or the peptide mixture at 10 μM of each peptide, with or without 1 U/ml recombinant IL-2 (rIL-2) (Amersham, Aylesbury, UK). PBMCs without peptide, with 2 μg/ml purified protein derivative of *Mycobacterium tuberculosis* (Veterinary Institute, Oslo, Norway) or with 1 μg/ml superantigen (SEC-3; Toxin Technology, Sarasota, FL) served as controls. Proliferation was assessed at day 7 after overnight incubation with ^{3}H-thymidine, 3.7×10^4 Bq/well (Amersham). This method was insensitive since only 4 patients had a positive response in this assay.

Therefore, all patients were additionally tested for T-cell responses after one restimulation *in vitro*. Briefly, PBMCs were seeded 1×10^6/well in 24-well plates supplemented with the immunizing peptide at 25 μM or the peptide mixture at 10 μM of each peptide in 1 ml of RPMI-1640 (GIBCO, Paisley, UK) containing 15% heat-inactivated human serum and antibiotics (referred to as R-15 medium). After 3 days of culture, the medium was supplemented with 10 U/ml rIL-2. Cultured cells (5×10^4/well) were tested after 9 to 12 days for specific proliferating capacity against single mutant ras peptides/peptide mixture and normal ras peptide at 25 μM concentration, with or without rIL-2 (1 U/ml), using autologous, irradiated (30 Gy) PBMCs (5×10^4 cells/well) as APCs. Proliferation was assessed at day 3 after overnight incubation with ^{3}H-thymidine, 3.7×10^4 Bq/well. Values are given as mean counts per minute (cpm) from 6 (proliferation assay) or 3 (after *in vitro* restimulation) determinations. Background responses (without antigen) were usually below 1,000 cpm and SD <10%. An antigen-specific response was considered positive when the stimulatory index (response with antigen divided by response without antigen) was above 2. Proliferating, peptide-specific T cells from responding patients were cloned by limiting dilution, as previously described.[14]

Expansion and testing of tumor-infiltrating lymphocytes

From some vaccinated patients with advanced disease, tumor biopsy specimens (fine needle histology) were obtained at the end of the study (week 14) and cultured for generation of tumor-infiltrating lymphocytes (TILs). Tumor biopsy samples of 6×1.2 mm diameter were placed directly in 1 well of a 24-well plate in 1 ml of R-15 medium supplemented with 100 U/ml rIL-2. Proliferating lymphocytes were allowed to grow out of the tumor biopsy specimen, and half of the medium was withdrawn and replaced

with fresh R-15 medium supplemented with 100 U/ml rIL-2 every third day. Developing cells were harvested at day 10; restimulated in R-15 medium supplemented with 1 μg/ml phytohemagglutinin (Wellcome, Dartford, UK), 100 U/ml rIL-2 and allogeneic irradiated (30 Gy) PBMCs (1×10^6/well) as feeder cells; and tested for peptide-specific proliferation after 5 to 7 days.

Cytotoxicity assay

Cytotoxicity of the $CD8^+$ T lymphocyte clone was measured in a 4 hr ^{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 hr at 37°C with gentle mixing every 15 min. Cells were washed 3 times in cold RPMI-1640, counted and seeded at 2×10^3 target cells in 96-well, U-bottomed microtiter plates. Target cells were pulsed with or without peptide for 1 hr at 37°C in a volume of 100 μl IMDM (Biowhittaker) or incubated with or without Cys12 mRNA and subjected to electroporation. Target cells were washed once in the microtiter plates before effector cells were added to a final volume of 200 μl in 15% human pool serum RPMI-1640. Maximum and spontaneous ^{51}Cr-release of target cells were measured after incubation with 5% Triton X-100 (Sigma, St. Louis, MO) 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. The percentage of specific chromium release was calculated by the following formula:

$$\frac{\text{(experimental release—spontaneous release)}}{\text{(maximum release—spontaneous release)}} \times 100$$

Toxicity criteria and clinical response

Patients were followed closely for signs of toxicity during and after vaccination. Adverse events were recorded using the WHO toxicity criteria. Tumor sites were evaluated by CT scans or ultrasonography before and at the end of the study period (week 14 for advanced tumor group, week 24 for resected patients). Tumor response (complete or partial) was evaluated according to accepted criteria (WHO). Stable disease was defined as a $<25\%$ change in tumor mass for more than 2 months, while progressive disease defined as a $\geq 25\%$ increase in tumor mass. Occurrence of ascites was also defined as disease progression. Performance status was evaluated according to the Karnofsky scale.

Statistical evaluation

Data from all patients who had received at least 1 vaccination were included in the analysis of safety. Analysis of efficacy (immunological response rate) was performed for all patients who had completed the trial according to the protocol or had received at least 4 peptide injections. The log-rank test was used to compare survival data (Kaplan-Meier plot) between the different groups of patients.

RESULTS

Safety

Peptide vaccination was well tolerated in all 48 patients and was administered on an outpatient basis. One patient reported headache lasting for some hours the mornings after the first and second vaccinations. Occasionally, mild fever or erythema around the vaccination site occurred, lasting 1 to 2 days. We observed no clinical signs of auto-immune disease or abnormal biochemical and hematological parameters related to the vaccinations. No sign of toxicity and no clinically important adverse events following peptide vaccination were observed. Thus, we conclude that mutant ras peptides in combination with GM-CSF could be repeatedly injected into patients without significant toxicity.

DTH reactivity/T-cell responses

Of 48 vaccinated patients, 43 were evaluable for induction of immunological response. DTH reactivity was tested at each vaccination and assessed 48 hr after each vaccination. A positive DTH reaction was observed in 21/43 patients (49%) at 1 or more visits during the study (Table III). DTH reactivity generally did not occur until the third vaccination, indicating efficient induction of mutant ras-specific T cells *in vivo.*

TABLE III – IMMUNOLOGICAL RESPONSE IN VACCINATED PATIENTS

	DTH reaction		Total
	Yes	No	
T-cell response			
Yes	13	4	17 (40%)
No	8	18	26 (60%)
Total	21 (49%)	22 (51%)	43

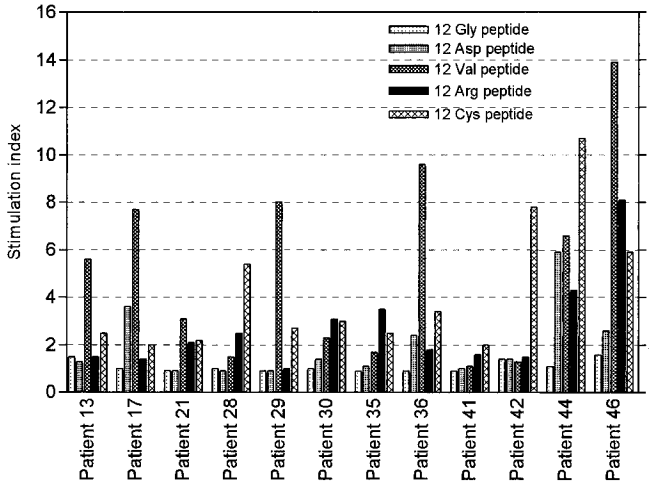

FIGURE 1 – Assessment of individual T-cell responses against the 4 synthetic mutant ras peptides contained in the vaccine cocktail in 12 responding patients. Post-vaccination PBMCs were stimulated *in vitro* with irradiated peptide-pulsed PBMCs and IL-2 as described in Material and Methods. Cultured cells were tested against peptide-pulsed PBMCs for specific proliferating capacity using ^{3}H-thymidine incorporation. Values are given as stimulation index for the 4 mutant ras peptides and the wild-type peptide.

We also examined DTH as an immunological end point in comparison with *in vitro* T-cell responses. Accordingly, all patients were tested weekly for proliferative T-cell responses against the immunizing peptide or the peptide mixture. In addition, after the fourth or fifth vaccination, T-cell reactivity was tested after 1 cycle of *in vitro* stimulation. The peptide vaccination elicited a positive T-cell response against mutant ras peptides in peripheral blood in 17/43 evaluable patients (40%) (Tables I–III). No patient showed any sign of *in vitro* T-cell responsiveness against the ras mutation before the onset of treatment (data not shown). Twenty-five patients (58%) demonstrated an overall immunological response to peptide vaccination, measured as either a DTH reaction or a T-cell response. The correlation between DTH response and T-cell response *in vitro* is shown in Table III: 13 patients showed both a DTH and a T-cell response, 4 patients showed a T-cell response but no DTH reaction and 8 patients demonstrated a DTH reaction but no *in vitro* T-cell response. From these data, we conclude that measuring DTH responses appears to be a simple and sensitive method for detecting an immune response to the vaccine.

Patients with advanced disease were vaccinated with a peptide cocktail, which consisted of a mixture of 4 different mutant ras peptides (codon 12: Asp, Val, Arg and Cys). The DTH test with the peptide cocktail did not reveal which of these peptides was responsible for the immune response. To investigate whether activated T cells from patients could recognize all 4 single compo-

nents of the peptide vaccine, post-vaccination PBMCs from the 12 responding patients (Table II) were tested against the 4 single-mutant peptides and the normal ras peptide after 1 stimulation *in vitro* (Fig. 1). Two of the patients mounted an immune response against all of the subcomponents of the vaccine, 4 reacted against 3 of the mutant ras peptides, 4 reacted against 2 of the mutant ras peptides and 2 generated an immune response against a single peptide. None of the patients showed cross-reactivity toward the normal ras sequence (Fig. 1).

Peptide-specific TILs

To investigate whether vaccine-specific T cells could be retrieved from the site of the tumor, fine needle biopsies were taken in 4 of the responding patients with advanced disease at the end of the protocol (week 14). Infiltrating lymphocytes were expanded *in vitro* with rIL-2 and tested for peptide-specific proliferation. In patient 13, who showed a partial response to therapy over an extended period of time, vaccination with mutant ras peptides and GM-CSF led to the proliferation of peptide-specific T cells in both peripheral blood and TILs cultured from a tumor biopsy specimen (Fig. 2). In the 3 other patients tested, we were not able to detect any peptide-specific TILs in the growing cultures from the biopsy specimen. In patient 13, we identified 2 different $CD4^+$ T-cell clones in peripheral blood that recognized the mutant ras peptides and expressed different TCRvβ families (1 $TCRv\beta17^+$ and 1 $TCRv\beta17^-$). FACS analysis showed that both clone 1.1B (generated from peripheral blood by peptide-driven clonal expansion) and the TILs from the tumor biopsy (driven by IL-2 expansion) were $CD4^+$ and $TCRv\beta17^+$ (Fig. 2*a*). Since both T-cell populations recognize all of the peptides in the vaccine mixture (Fig. 2*b*), including the Arg12 mutation found in the tumor, we conclude that peptide vaccination in this case resulted in expansion of a cross-reactive T-cell clone capable of recognizing the mutation. T cells from this clone were selectively enriched in the tumor since we found no evidence for enrichment of a second T-cell clone (clone 1.27 specific for the Val12 mutation not present in the tumor). In this patient, vaccination with mutant ras peptides resulted in the induction of peptide-specific T cells *in vivo* that can home to the site of the tumor. This is of major concern since not only the induction of peptide-specific T cells but also the co-localization of such T cells to the tumor target area is necessary for vaccination strategies to be successful/clinically effective.

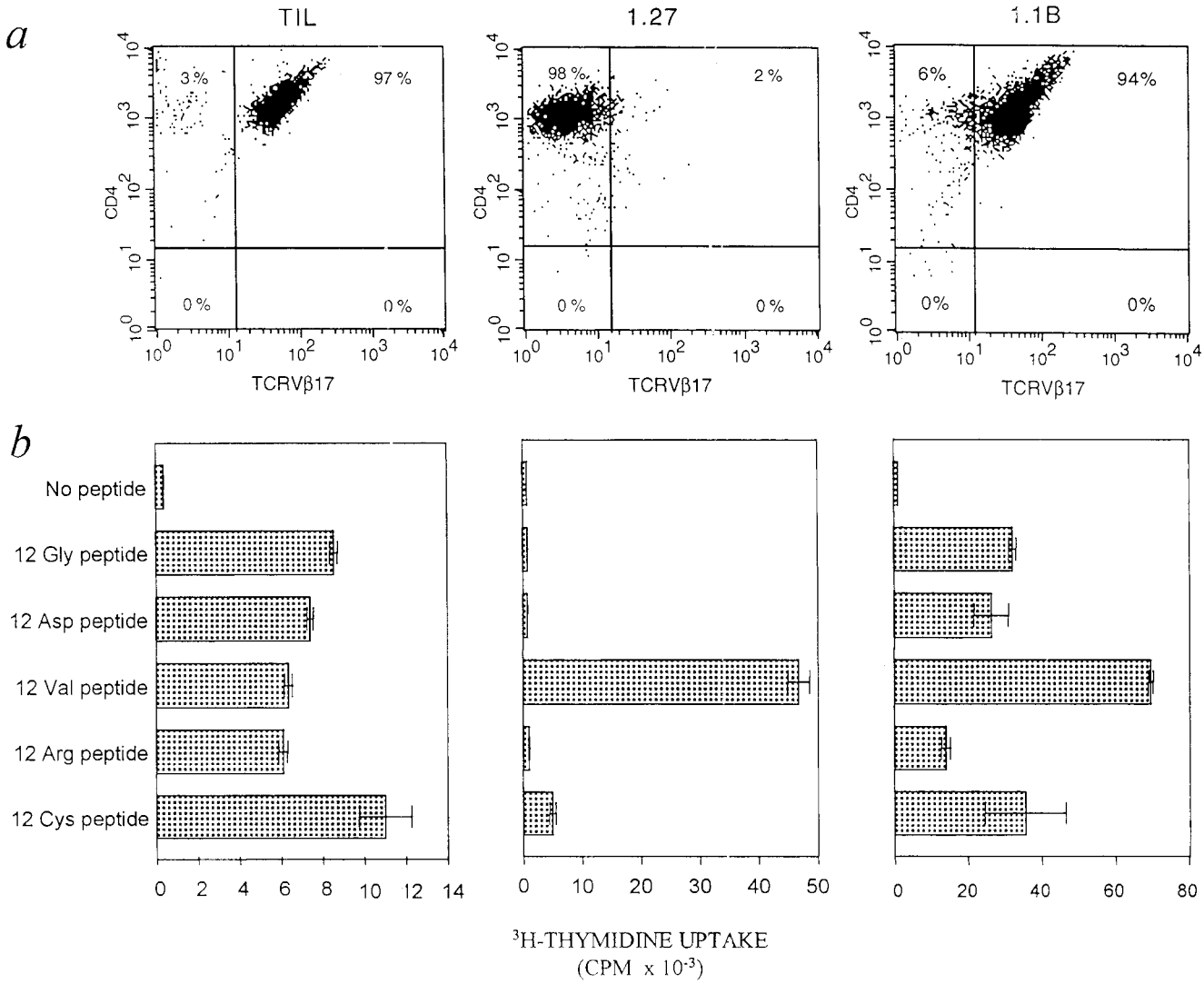

FIGURE 2 – Recruitment of peptide-specific T cells into the tumor site in patient 13. *(a)* Flow cytometry of TILs and peripheral T-cell clones (1.27 and 1.1B) from patient 13. TILs and clone 1.1B show co-expression of CD4 and TCRVβ17, whereas clone 1.27 did not express TCRVβ17. *(b)* Peptide-specific proliferation of TILs and peripheral T-cell clones 1.27 and 1.1B. TILs and peripheral T-cell clones were tested for peptide specificity in the presence of irradiated, peptide-pulsed PBMCs.

Detailed monitoring of single patients

Some patients displaying an immune response to the vaccine and a favorable clinical evolution were given additional vaccinations. A detailed follow-up showing the duration of the induced immunity in 4 of the responding patients is shown in Figure 3. These 4 patients displayed long-lived immunity to the peptide vaccine; in patient 1, persistent T-cell immunity could be demonstrated for up to 8 months. Patient 1, who had her pancreatic cancer resected in January 1997, displayed a positive DTH reaction after the fourth vaccination, and T cells proliferating to the Val12 peptide were detected in circulation after the fifth vaccination and persisted for 2 months, whereupon they were no longer detectable. The patient received a follow-up vaccination 8 months after the first vaccination cycle. A positive DTH response was seen 48 hr after the first peptide injection, indicating the presence of long-term memory T cells. Specific T cells could be detected in the circulation 1 week later and persisted throughout the second vaccination cycle. Three months later, the patient received a third vaccination cycle and both a DTH reaction and Val12-reactive circulating T cells could be detected. Taken together these observations indicate that long-term immune responses can be obtained using the present protocol. The data also illustrate that repeated injections are required to maintain T-cell responsiveness at a certain level

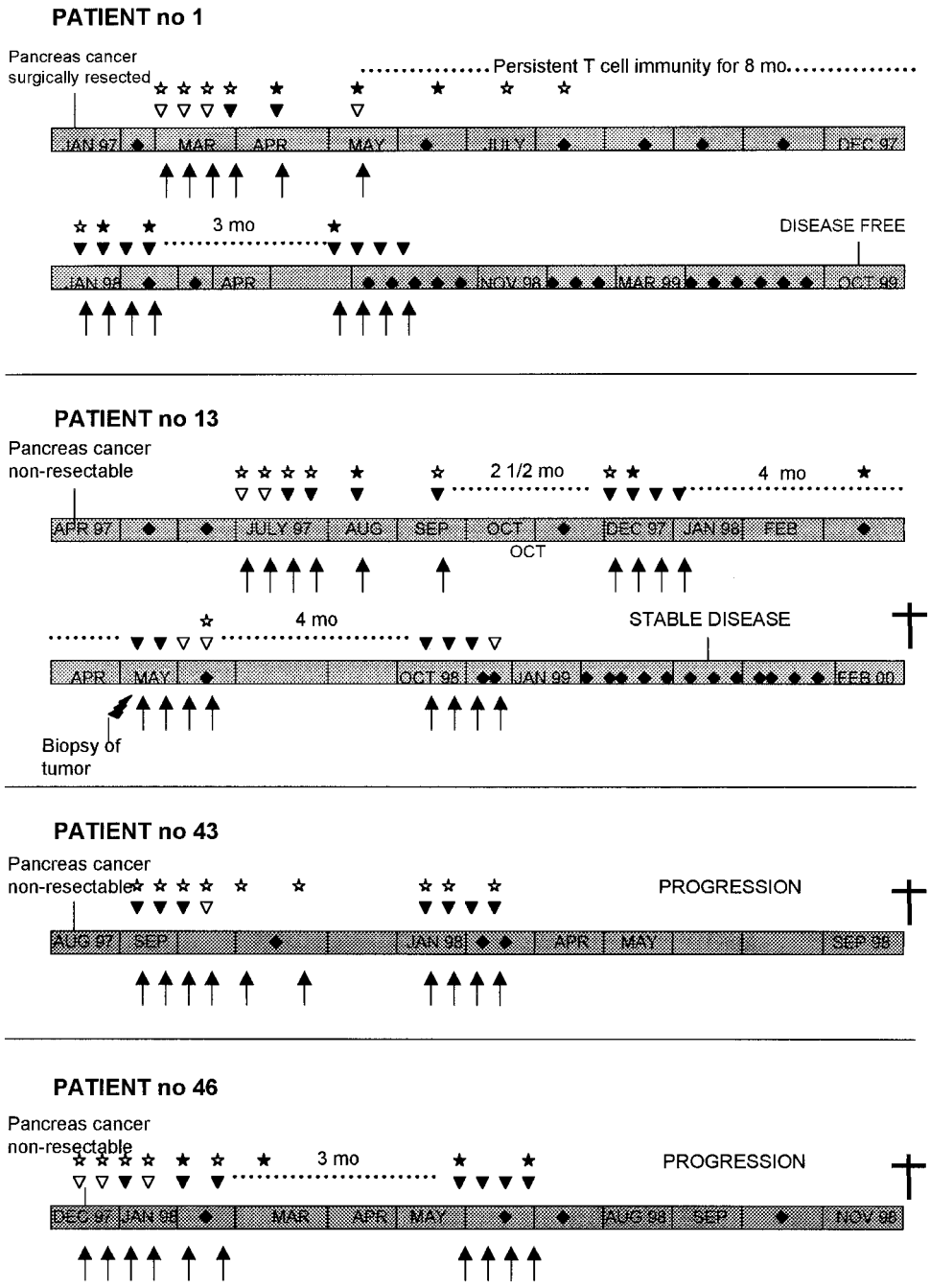

FIGURE 3 – Detailed monitoring of 4 immunologically responding patients. Patient 1 had no detectable disease and patients 13, 43 and 46 had advanced pancreatic cancer. Symbols: upward arrow, peptide vaccination; downward solid arrowhead, DTH positivity; downward open arrowhead, DTH negativity; solid star, peripheral T-cell positivity; open star, peripheral T-cell negativity; solid cross, death.

and that monitoring immune responses *in vitro* is less sensitive than monitoring DTH reactivity.

Patient 13 had a non-resectable pancreatic cancer diagnosed in April 1997. She developed a positive DTH reaction after the third peptide injection and a positive peripheral T-cell response after the fifth injection. At this stage, T cells were cloned from peripheral blood (Fig. 2). Subsequently, the patient received 3 additional cycles each of 4 weekly injections. Long-lived T-cell immunity was evident from a positive DTH response observed at the start of each vaccination cycle (Fig. 3). Again, the peripheral blood T-cell response was less reliable as a parameter for monitoring response to the vaccine since it fluctuated throughout the vaccination period. One important observation in this patient was the loss of DTH reactivity in connection with the third and fourth injections in the last 2 vaccine cycles. This may have been due to competition for antigen-specific cells between the tumor site, the inflammatory vaccination site and the DTH site, with preferential retention at the 2 former sites. This observation may have implications for the design of T-cell monitoring in vaccine protocols since a negative reaction would have erroneously been concluded in protocols where DTH is measured only in connection with the last injection. Evidence for retention of specific cells at the tumor site was obtained from a biopsy taken in May 1998, when T cells specific for the vaccine could be isolated (Fig. 2).

Patient 43, who had a non-resectable tumor, showed unique reactivity by manifesting a positive DTH reaction 48 hr after initiation of the first vaccination cycle. This indicated that the patient had pre-existing immunity against 1 of the vaccine components. Unfortunately, we were unable to demonstrate a T-cell response against any of the vaccine components *in vitro* in this patient, even after the patient had undergone a booster vaccine cycle. Thus, we could not confirm that the patient had been primed against the Val12 mutation present in his tumor.

Patient 46 had a non-resectable tumor diagnosed in December 1997. Following the first vaccination cycle, the patient developed a long-lasting immune response, which was detectable both at the level of DTH reactivity and *in vitro* when the patient received a booster vaccination cycle 3 months following the first cycle. Cloning of the responding T cells showed $CD4^+$ T cells specific for the Val12 mutation present in the tumor. In addition, T-cell clones specific for other ras mutations, including a $CD8^+$ T-cell clone specific for Cys12 could be isolated. The latter T-cell clone was derived from an *in vitro* T-cell culture stimulated with the helper epitope KLVVVGACGVGKSALTI. This CTL clone recognized a nested nonamer epitope containing the Gly12→Cys substitution (data not shown). No tumor cell lines that co-expressed both the *K-RAS* mutation Gly12→Cys and the appropriate HLA molecule were available from cell banks, and we were not able to obtain autologous tumor samples from the patient. We therefore constructed surrogate tumor targets by mRNA transfection of autologous B-LCLs with the ras Cys12 mutation (Fig. 4). Surrogate target cells were killed as efficiently as peptide-pulsed target cells. This demonstrates that the peptide-induced $CD8^+$ T-cell clone can also recognize a processed form of the corresponding mutant ras protein and indicates that such $CD8^+$ CTLs may be part of the effector machinery generated by vaccination with these promiscuous helper epitopes.

Clinical response/survival

In the group of radically resected patients, 1 was withdrawn after 3 vaccinations due to disease progression, whereas the remaining 9 had stable disease (Table I). A follow-up report on these patients will be presented after a longer period. Mean survival in this group (at the time of this writing) was 25.6 months (range 10–39 months) *vs.* 16.7 months for historical controls.[26]

In the group with non-resectable cancer, 11/34 evaluable patients (32%) had stable disease after peptide vaccination (Tables II, IV) and all of the patients with stable disease showed an immunological response (Table IV). Nine of 20 patients (45%) showing

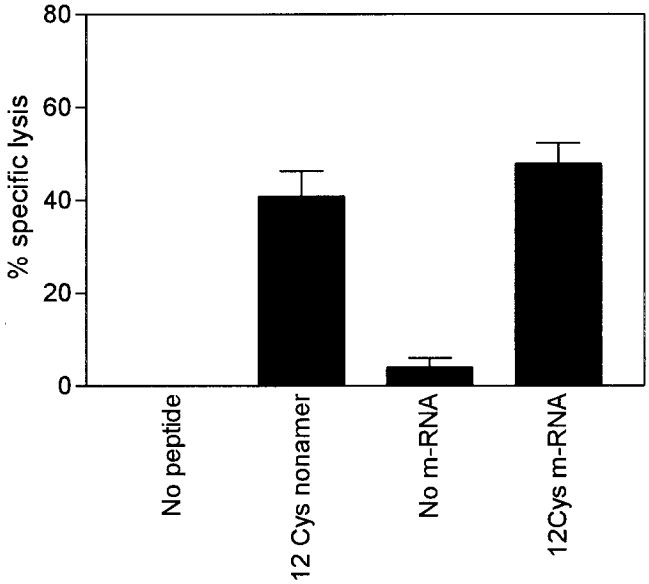

FIGURE 4 – Cytotoxicity against *K-RAS* Gly12→Cys-expressing target cells by a $CD8^+$ T-cell clone isolated from patient 46. Target cells (autologous B-LCLs) were transfected with full-length mRNA for *K-RAS* Cys12. Untransfected cells were used as control. Cytotoxic activity was also examined against autologous B-LCL cells pulsed with the 9 mer Cys12 peptide or against B-LCL cells without peptide. Final peptide concentration was 10 nM. Data represent percent specific lysis of Cr-labeled targets in a 4 hr assay and are expressed as means ± SD of triplicate cultures.

TABLE IV – TUMOR RESPONSE COMPARED WITH OVERALL IMMUNOLOGICAL RESPONSE IN PATIENTS WITH ADVANCED DISEASE

	Immunological response		Total
	Yes	No	
Tumor response[1]			
SD	11[2]	0	11 (32%)
PD	9	14	23 (68%)
Total	20 (59%)	14 (41%)	34

[1]Clinical response: SD, stable disease; PD, progressive disease.– [2]One of these patients is still alive (at the time of this writing), with a survival time from the start of vaccination of 23 months (patient 29).

an immune response to the vaccine had progression of disease, while all immunological non-responders exhibited disease progression (Table IV). The 11 immunologically responding patients with stable disease had a median duration of clinical response lasting 10.2 months (range 3–28 months) (Table II). In patient 13, demonstrating a partial clinical response to peptide vaccination, regression of a pancreatic tumor is shown after 3 cycles of vaccination (Fig. 5). The tumor area in the pancreas was less prominent after the vaccine treatment (Fig. 5*b*), which was in accordance with the clinical condition of the patient. This patient continued to have a favorable clinical outcome and was still in good condition after a follow-up period of 28 months. Then, her clinical condition gradually deteriorated until she died 31 months from the start of treatment.

Generation of an immune response against the vaccine was associated with longer survival (Fig. 6*a*). Median survival in responders was 148 days and in non-responders, 61 days. The difference in survival time between the immunological responders and non-responders was statistically significant ($p = 0.0002$) (Fig. 6*a*). There was no correlation between immune status (based on the ability to respond to superantigens) at the start of vaccination and survival time ($p = 0.72$) (Fig. 6*b*). Furthermore, there was no correlation between immune status at the onset of vaccination and

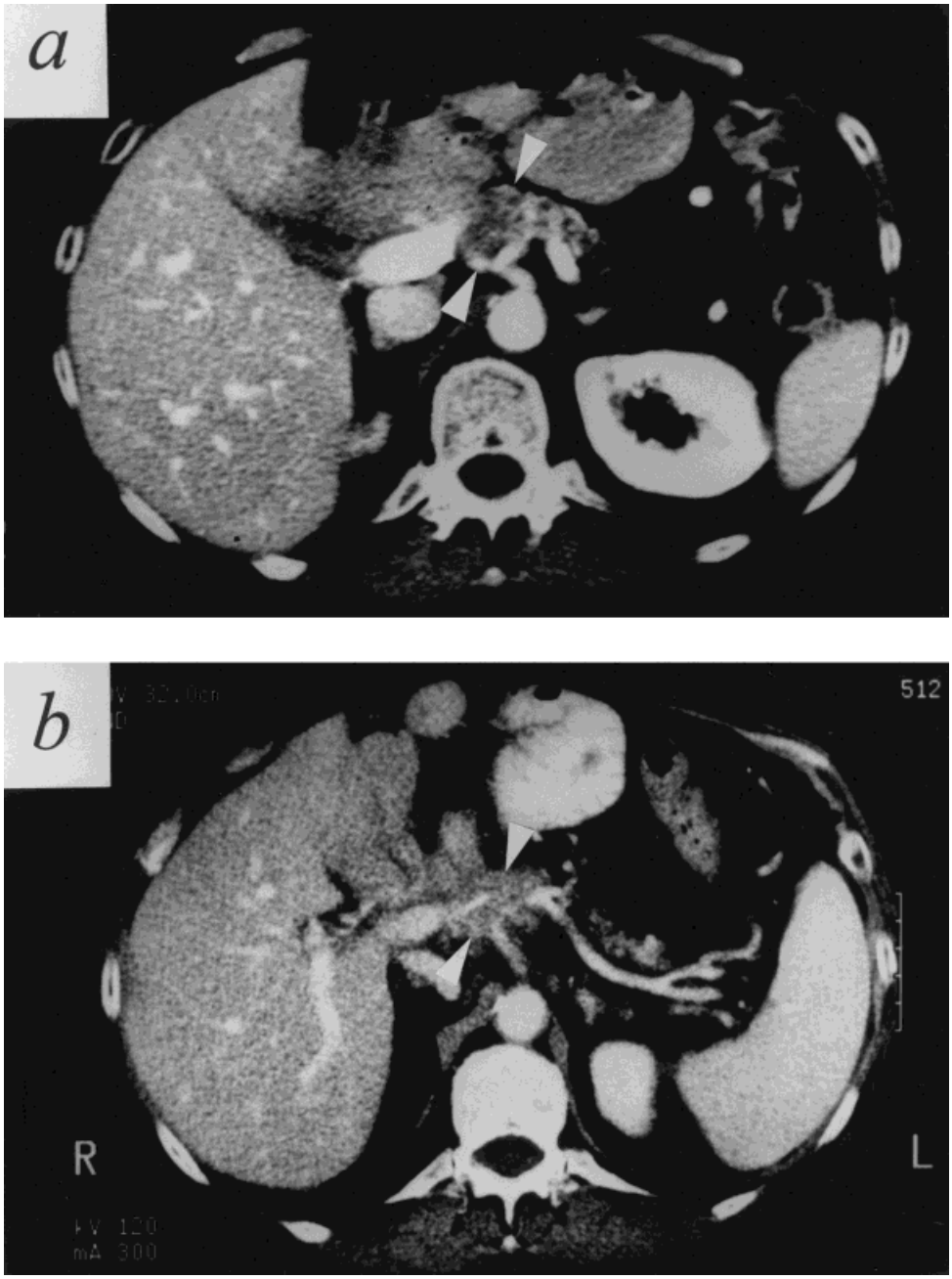

FIGURE 5 – Regression of a non-resectable pancreatic tumor in a 63-year-old female patient (patient 13 in Table II). CT scan of the pancreas before *(a)* and after *(b)* 3 cycles of vaccination with mutant ras peptides. Tumor area (white arrowheads) is less prominent after vaccination with mutant ras peptides. After 28 months of follow-up, the patient was still in a good clinical state, showing stable disease.

response to the peptide vaccine (data not shown). These data show that induction of ras-specific immunity in pancreatic cancer patients with advanced disease is significantly associated with an enhanced survival time compared to patients, which do not generate an immune response toward mutant ras.

DISCUSSION

Several different vaccination strategies to generate anti-tumor activity are currently being investigated in cancer patients. Most clinical trials have involved patients with advanced melanoma. Similar immunotherapeutic strategies have been investigated in patients with advanced adenocarcinoma; thus, 2 phase I studies have demonstrated the feasibility of mutant ras peptide vaccination in patients with advanced cancer.[11,12,27] We demonstrated that vaccination with autologous APCs loaded with the relevant mutant ras peptide could induce peptide-specific T-cell responsiveness *in vivo* in 2/5 vaccinated pancreatic cancer patients.[11,27] The 2 responding patients showed a transient immune response that was detected only due to frequent monitoring of the T-cell response, indicating that the vaccination protocol was suboptimal. Also, responding patients survived for a prolonged period of time. These preliminary results prompted us to look for better vaccination procedures and more sensitive ways of monitoring immune responses. Another study in patients with metastatic adenocarcinoma demonstrated that s.c. vaccination with Detox (Ribi ImmunoChem

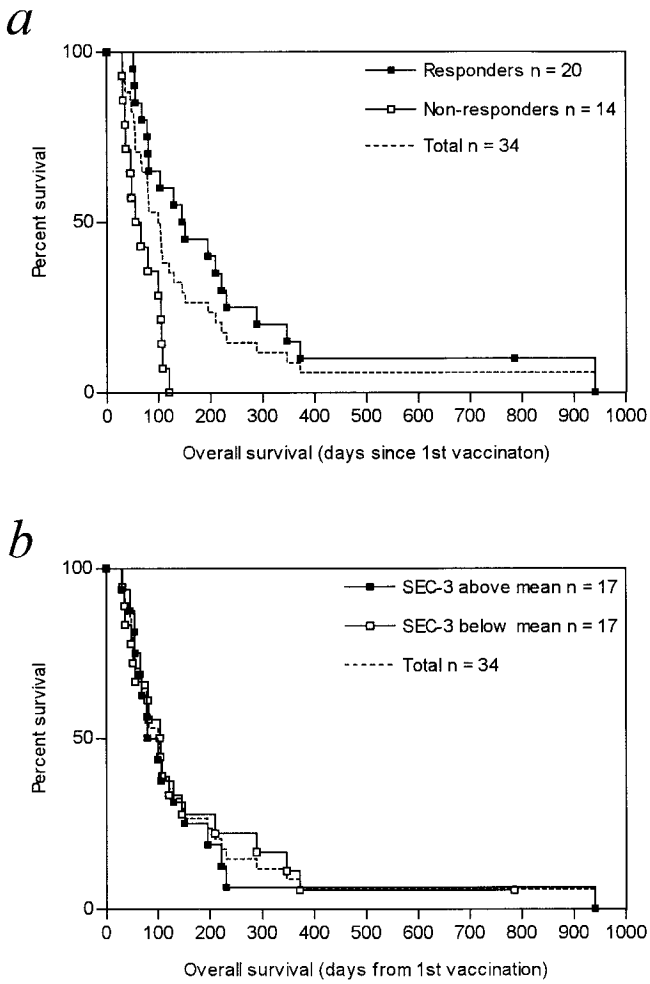

FIGURE 6 – Overall survival of patients with advanced pancreatic cancer after vaccination with mutant ras peptides. *(a)* Patients with an immune response to the peptide vaccine have a significantly longer survival time compared with those not generating an immune response to therapy ($p = 0.0002$). *(b)* Comparison of survival time in the 2 groups based on ability to respond to superantigen *in vitro* at the beginning of the study. Survival time from the start of vaccination is not significantly different in the group with reactivity to SEC-3 above the mean (median survival 89 days) compared to the group with reactivity to SEC-3 below the mean (median survival 103 days) ($p = 0.72$).

Research, Inc., Hamilton, MT) adjuvant mixed with the appropriate mutated ras 13-mer peptide led to the induction of peptide-specific T-cell responses in 3/8 evaluable patients.[12,15] No objective clinical response was observed in this study.

In the present study, we report that i.d. vaccination of pancreatic cancer patients with synthetic mutant ras peptides with GM-CSF as an adjuvant is highly effective at generating high-level ras peptide-specific T-cell responses. The majority of patients had advanced pancreatic cancer with expected survival of only 3 to 4 months. Even with this background, we were able to elicit immune responses in 58% of the evaluable patients. With 1 exception, none of the patients demonstrated ras peptide-specific immunity prior to vaccination. Immune responses were long-lasting and improved after repeated cycles of vaccination. In a tumor biopsy taken after vaccination, we demonstrated the presence of T cells specific for the ras mutation expressed by tumor cells. A significant association between prolonged survival and immune response to the vaccine was also observed. No sign of toxicity and no clinically important adverse events following the treatment were observed. Only a few, mild adverse events were reported, probably related to the injection of GM-CSF.

Our data support the results of Jaeger *et al.*,[22] who found enhanced DTH and CTL responses after i.d. peptide immunization in combination with systemic GM-CSF administration. Disis *et al.*[23] reported similar results using the same vaccination strategy to generate immune responses against Her-2/neu peptides in patients with ovarian or breast cancer. Together these studies confirm that GM-CSF is a potent adjuvant in human cancer vaccine trials. Our vaccination protocol was greatly simplified compared to other protocols using GM-CSF since only a single i.d. injection, rather than a 4- to 6-day period of s.c. injection, was used.

In general, a correlation was seen between a positive DTH response and a T-cell response *in vitro;* however, in 8/21 patients with a DTH response, no T-cell response could be observed. DTH responses represent a 48 hr sampling of specific T cells from the circulation, while blood was drawn for *in vitro* testing within a very narrow time frame. This may explain the higher sensitivity of the DTH response. In 4 patients, we observed *in vitro* T-cell responses in the absence of DTH response. Since the DTH reaction is thought to represent a Th1-type response, these patients may have generated a Th2-type response. This possibility was not investigated in the present study.

Vaccination with a mixture of homologous peptides could potentially result in immunodominance due to competition for the same HLA class II molecules. In our study, induction of responses to all of the 4 peptides contained in the vaccine preparation was evident in several patients, indicating that immunodominance is not a major problem. Similar results have been observed in 2 other clinical trials using mixtures of ras peptides and GM-CSF as adjuvant (unpublished results).

Nestle *et al.*[20] reported a good correlation between the induction of peptide-specific DTH reactivity *in vivo* and clinical responses. In that study, vaccination of melanoma patients with advanced disease with peptide or tumor lysate–pulsed DCs led to induction of peptide-specific DTH reactivity in all patients treated, which was associated with a clinical response in 6/16 patients (38%). In another study, where patients with metastatic melanoma were vaccinated with synthetic peptides from the gp100 melanoma-associated antigen in combination with IL-2, 13/31 patients (42%) experienced objective tumor response.[28] In the present study, we also observed an association between induction of a vaccine response and prolonged survival of responding patients. Patients with advanced disease showing an overall immunological response after peptide immunization demonstrated a strong trend to live longer than those who did not show an immune response. This trend was not observed when we tried to correlate survival with cellular immune status at inclusion. This finding confirms the observation made in our pilot study.[11] The majority of patients had advanced pancreatic disease and a rapidly deteriorating immune system, eventually resulting in complete lack of reactivity to strong T-cell stimulators, such as superantigens (data not shown). Our study demonstrates clearly that despite their advanced clinical state and emerging immunosuppression, the majority of patients were still able to elicit immune responses against the ras peptide vaccine. Surprisingly, this response was associated with prolonged survival. One explanation for this may be that patients who respond are biologically more fit to fight their cancer and that the association is not related to the response to the ras vaccine *per se.* The finding that patients demonstrating the highest response to superantigens did not display evidence for better biological fitness when compared with "low" responders may argue against this explanation. An alternative explanation, that induction of ras mutation-specific T cells is responsible for increased survival in this group, is provocative. In 1 of our patients, we have, however, shown directly that initiation of a specific T-cell response against the mutation expressed in the tumor resulted in accumulation of the relevant T cells in the tumor and that this was associated with a good clinical course. Even though we did not formally demon-

strate the lack of these TILs in a tumor biopsy taken before vaccination, we did not find such reactivity in blood taken prior to vaccination. However, after vaccination, T cells with the same fine specificity and TCRVβ17 could be isolated from both peripheral blood and the tumor. This means that even if we presume that these cells were trapped in the tumor before vaccination, the vaccination procedure resulted in expansion of the specific T-cell clone(s) followed by the appearance of cells in the circulation. Thus, it is reasonable to believe that these cells, which are activated and clonally expanded following vaccination, have the capacity to home to the tumor site.

Although ras-specific helper T cells may generate a cascade of events *in situ* in the tumor and ras-specific CTLs may efficiently kill tumor cells, single-epitope vaccination is probably not enough to eradicate a large body of tumor cells. This is evidenced by the fact that the majority of patients who gave rise to an immune response died of their disease. We therefore believe that adding other epitopes in combination with additional orchestration of the immune response by sequential use of cytokines to augment both the induction phase and the effector phase of the immune response will be required to obtain better clinical responses. Furthermore, emphasis must be put on ways to reverse the immunosuppressive environment which meets the activated T cells that make their way to the tumor.

In conclusion, vaccination with mutant ras peptides in combination with GM-CSF in patients with pancreatic cancer is well tolerated and can induce anti-tumor immunity *in vivo.* In patients with advanced disease, measurable DTH reactivity and/or T-cell responsiveness is associated with prolonged survival time. These results are encouraging, and randomized trials should be carried out to evaluate the efficacy of ras peptide vaccination in combination with GM-CSF.

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