Characterisation of immune responses in pancreatic carcinoma patients after mutant p21 ras peptide vaccination

MK Gjertsen, I Saeterdal, E Thorsby and G Gaudernack

Institute of Transplantation Immunology, The National Hospital, University of Oslo, N-0027 Oslo, Norway.

Summary This is a study of immune responses generated by mutant ras peptide vaccination of patients with pancreatic adenocarcinoma. Responding T cells from one patient were cloned and two CD4+ T-lymphocyte clones (TLC) specific for the 12 Val peptide and restricted by HLA-DR6 or DQ2 were obtained. These class II molecules have not previously been found to bind or present mutant ras peptides to T cells. The DR6-restricted TLC showed marked cytotoxicity against autologous target cells pulsed with the 12Val peptide. Target cells pulsed with the control peptide were not killed. Responding T cells from another patient showed cross-reactivity to the homologous ras peptides. Investigation by limiting dilution analysis (LDA) revealed different T-cell precursor frequencies for the immunising, mutant ras peptide (1:28 000), compared with the normal ras peptide (1:110 000).

Keywords: mutant p21 ras; T cells; peptide vaccination; pancreatic adenocarcinoma

Somatic point mutations of K-ras oncogenes occur in approximately 90% of pancreatic adenocarcinomas (Almoguera et al., 1988). Activating point mutations of K-ras oncogenes predominantly occur at codons 12, 13 or 61 and result in corresponding single amino acid substitutions within the p21 protein. In pancreatic adenocarcinomas, the mutations most commonly occur at codon 12 of K-ras, and the spectrum of different amino acid substitutions is limited (Capella et al., 1991). The mutations disrupt the normal signalling function of p21 ras and contribute to malignant transformation (Seeburg et al., 1984; Der et al., 1985).

Mutant p21 ras are not expressed by normal tissue and thus represent true tumour-specific antigens. Mutant ras proteins or corresponding peptide sequences have previously been shown to be immunogenic both in healthy individuals (Jung and Schluesener, 1991) and in cancer patients (Gedde-Dahli et al., 1992a; Fossum et al., 1994). Most responding T cells have been of the CD4+ phenotype, and their peptide specificity has been described in detail (Gedde-Dahli et al., 1993). Ras peptide binding to HLA class II molecules seems to be promiscuous (Gedde-Dahli et al., 1994; Johansen et al., 1994) and involves class II molecules of all isotypes (Fossum et al., 1993). Also, human CD8+ TCLs specific for a single ras mutation and capable of killing tumour cells harbouring the same mutation have been described (Fossum et al., 1995). Together these results suggest that ras peptide vaccination of cancer patients with a verified ras mutation, might be applied as a therapeutic principle of specific immunotherapy.

In a pilot clinical study, we have vaccinated pancreatic carcinoma patients with synthetic ras peptide-pulsed autologous antigen-presenting cells (APCs), and thereby induced peptide specific T-cell responsiveness in vivo (Gjertsen et al., 1995, 1996). To characterise the specificity further, HLA restriction and functional properties of these in vivo activated T cells, TCLs were generated from one of the vaccinated patients. We report here that the vaccination procedure resulted in a sufficient clonal expansion of CD4+ T cells specific for the Gly12→Val mutation to allow detection in peripheral blood and subsequent cloning. The HLA class II molecules involved in binding and presenting of these synthetic ras peptides to T cells in vivo were identified in antibody-blocking experiments and by using a panel of HLA-homozygous B-lymphoblastoid cell lines (B-LCLs) as APC.

We also provide evidence that one of the CD4+ TCLs has cytotoxic properties and is capable of specifically killing autologous target cells pulsed with the appropriate ras peptide. Different T-cell precursor frequencies against mutant and normal ras peptides in peripheral blood were determined by LDA.

Materials and methods

T-cell donors

The male patient (donor 1), 49 years old at diagnosis, had a moderately differentiated, unresectable adenocarcinoma of the pancreatic head. The HLA type of donor 1 was HLA-A1,2; B6,35; DR3,6 (DBR1*0301,1401); DQ1,2 (DQA1*0101,0501;DQB1*0201,0501). The K-ras mutation Gly12→Val was identified in DNA from formalin-fixed paraffin-embedded tumour tissue using a highly sensitive technique (Kahn et al., 1991). The female patient (donor 2), 39 years old at diagnosis, had a poorly differentiated, unresectable adenocarcinoma of the pancreatic head with one liver metastasis. The HLA type of donor 2 was HLA-A3,19; B7,12; DR4,7; DQ2,3. The K-ras mutation in tumour tissue from donor 2 was found to be Gly12→Asp. Both patients were vaccinated with autologous ras peptide-pulsed APC [freshly isolated, unfractionated peripheral blood mononuclear cells (PBMCs)], and two and three rounds of vaccination induced proliferative T-cell responses in PBMCs of donor 1 and donor 2 respectively (Gjertsen et al., 1995).

Cells and media

PBMCs were prepared by centrifugation over Lymphoprep (Nycomed, Oslo, Norway). B-LCLs used as APC were from the 10th and 11th International Histocompatibility Workshop cell panels. The HLA profiles of the different cell lines used are given in Table I. Autologous B-LCLs were generated by Epstein–Barr virus transformation of B cells from the patients. The tumour cell line K562 (erythroleukaemia) was used as control cells in cytotoxicity assays. The IL-2-dependent murine cytotoxic cell line CTL2-2 (Gillis et al., 1978) was used as indicator cell line in LDA. All cultures were grown in RPMI-1640 (Gibco, Paisley, UK) supplemented with gentamicin, 15% heat-inactivated human pool serum (T cells) or 10% heat-inactivated fetal calf serum (FCS) (Gibco) (cell lines). For the CTL2-2 cell line, we additionally used human recombinant interleukin 2 (rIL-2) 5 U ml⁻¹ (Amersham, Aylesbury, UK).
Peptides

Peptides encompassing residues 5–21 of p21 ras, KLVVGAGGYGKSLTIG (single letter code), or with a Gly to Val, Arg, Asp or Cys substitution at residue 12 or a Gly to Asp substitution at residue 13, were synthesised and purified as described earlier (Gedde-Dahl et al., 1992b). The ras peptide spanning position 51–67 of p21 ras, CLLDILD-TAGQEEYSAM, was used as a control in some experiments. The peptides were dissolved in sterile water before filter sterilisation.

Antibodies

Standard monoclonal antibodies (MAbs) against non-polymorphic determinants of HLA class II were used: SPV-L3 (anti-HLA-DQ) (a gift from Dr H Spits, Palo Alto, CA, USA); B8.11 (anti-HLA-DR) (a gift from Dr B Malissen, Marseille, France) and B7.21 (anti-HLA-DP) (a gift from Dr F Bach, University of Minnesota, Minneapolis, MN, USA). MAbs used for phenotyping of CD4+ (66.1) and CD8+ (ITI-SC2) TCLs were used as described earlier (Gaudernack and Lundin, 1989).

Generation of T-cell clones

Responding PBMCs from donor 1 were plated 2 × 106 cells per well in 24-well plates (Costar, Cambridge, MA, USA) and stimulated with 12Val peptide at 25 μM and rIL-2, 5 U ml−1 (Amersham). After 9 days, TLCs were transferred onto flat-bottomed 96-well plates (Costar) with 1 μg ml−1 phytohaemagglutinin (PHA, Wellcome, Dartford, UK), 5 U ml−1 rIL-2 and allogeneic, irradiated (30 Gy) PBMCs (2 × 106) per well as feeder cells. After 6 days, TCLs were transferred to 24-well plates with PHA/rIL-2 and 1 × 106 allogeneic, irradiated PBMCs as feeder cells and screened for peptide specificity after 4–7 days.

Proliferative assay

In proliferative assays, B-LCLs used as APCs were irradiated (100 Gy) and seeded (5 × 105 cells per well) in 96-well U-bottomed microtitre plates (Costar). Peptides were added at a final concentration of 15 μM and the cells were incubated for at least 2 h at 37°C before addition of T cells (2–5 × 105). In antibody-blocking experiments, APCs were incubated with MAbs for 1 h at 37°C before addition of T cells. Final concentrations of MAbs were 10 μg ml−1. Proliferation was measured at day 3 after co-incubation with 3.7 × 106 Bq [3H]thymidine (Amersham) 18 h before harvesting. Values are given as mean counts per minute (c.p.m.) from triplicates ± standard deviation (s.d.). An antigen-specific response was considered positive when the stimulatory index (SI) (i.e. response with antigen divided by the response without antigen) was above 3.

Cytotoxicity assay

Autologous B-LCLs were pulsed overnight with peptide at a concentration of 25 μM, then washed and labelled with 7.5 MBq 51Cr and FCS in a total volume of 0.5 ml at 37°C for 1 h, with gentle shaking every 15 min. Target cells were washed three times, and seeded (2 × 103 cells per well) in 96-well U-bottomed microtitre plates (Costar). Effector cells were added at different numbers as indicated. In antibody-blocking experiments, target cells were incubated with MAbs for 30 min at 37°C before addition of T cells. MAbs were used at a final concentration of 10 μg ml−1. Maximum and spontaneous 51Cr release of target cells was measured after incubation with 5% Triton-X or medium respectively. Supernatants were harvested after 4 h incubation at 37°C and radioactivity was measured by gamma spectrometry (Wallac 1470 Wizard). Percentage of specific chromium release was calculated by the formula:

\[
\text{Percentage of specific chromium release} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100
\]

Spontaneous release was always below 20% of the maximum release.

Limiting dilution analysis

The limiting dilution microculture conditions were adapted from a method of IL-2 detection described previously (Orosz et al., 1987). Briefly, irradiated (30 Gy) PBMCs (1 × 105) were plated in replicate 96-well U-bottomed microtitre plates (Costar) and allowed to adhere. After incubation for 2 h at 37°C, the plates were washed twice to remove non-adherent cells. The washed, adherent cells (stimulator cells) were pulsed with peptides at a concentration of 25 μM before addition of PBMCs (responder cells) in replicate sets of 12 microwells for each of seven, 2-fold serial responder cell dilutions starting with 5 × 104 cells per well. After 24 h of incubation at 37°C in 5% carbon dioxide, CTLL-2 cells (2 × 105) were added to each well. After another 18 h, the wells were pulsed with 3.7 × 106 Bq [3H]thymidine (Amersham) for 6 h before harvesting. Estimates of the T-cell precursor frequencies were calculated by analysis of the Poisson distribution relationship between the number of responder PBMCs added and the percentage of replicates

| APC | DRB1 | HLA class II | DQ81 | DQ82 | TLC 42-4 | TLC 69-29 |
|-----|------|-------------|------|------|----------|----------|
| EK  | 0904 | *1401       | *0101| *0503| 21116    | 1001     |
| TEM | 0907 | *1401       | *0101| *0503| 21116    | 1001     |
| 31227ABO | 0906 | *1401       | *0101| *0503| 21116    | 1001     |
| VAVY | 0923 | *0301       | *0501| *0201| 720.0    | 7327.0  |
| STEINLIN | 0907 | *0301       | *0501| *0201| NT       | NT       |
| PF0015 | 0908 | *0301       | *0501| *0201| NT       | NT       |
| BOLETH | 0903 | *0301       | *0302| *0302| 1519     | 7384.0   |
| OMW | 0908 | *1301       | *0103| *0603| 1587     | 1183.0   |
| WT47 | 0906 | *1302       | *0102| *0604| 1391     | 1289.0   |
| EBD | *0301*1401 | *0101*0501| *0201*0503| 17695 | 55          | 40048 |
wells that failed to produce detectable IL-2 (Taswell et al., 1981). Wells were considered positive for IL-2 production when 

\[ {}^{[3]H} \text{thymidine incorporation} \]

exceeded the mean of the background plus three s.d.

**Results**

**Specificity and HLA restriction of T-lymphocyte clones**

In donor 1, a proliferative T-cell response against the ras-derived 12Val peptide was induced after two rounds of vaccination with peptide-pulsed autologous APC (Gjertsen et al., 1995). The proliferative T-cell response in PBMCs was detected in a standard 7 day proliferation assay. Responding T cells were cloned by limiting dilution from a bulk culture initiated after the proliferative response was observed, and several TLCs of both the CD4+ and CD8+ phenotype were obtained. TLC 42-4 is CD3+, CD4+, TCR \( \alpha\beta \) and specific for the 12Val peptide (Figure 1). Antibody-blocking studies revealed that TLC 42-4 was HLA class II restricted, and that the 12Val peptide was presented by HLA-DR, since MAbs against DR but not DQ or DP abolished the response (Figure 1). Donor 1 was heterozygous for DR and genomic HLA-typing showed that DRB1*0301,1401 was present. By employing a panel of homozygous B-LCLs as APC, DRB1*1401 was identified as the restriction element of TLC 42-4 (Table I). Another TLC 69-29 generated from donor 1 after four rounds of peptide vaccination, is also CD3+, CD4+, TCR \( \alpha\beta \) and specific for the 12Val peptide (Figure 2). Studies with MAbs identified HLA-DQ as the antigen-presenting molecule of TLC 69-29, since MAbs against DQ and not DR or DP abolished the response (Figure 2). Panel studies using homozygous B-LCLs identical to DQA1 and DQB1 of donor 1 showed that DQB1*0201 is the restriction molecule of TLC 69-29 (Table I).

**Cytotoxicity of CD4+ T cells against peptide-pulsed EBV-transformed B cells**

To determine if the TLC 42-4 from donor 1 could lyse autologous B-LCLs presenting the immunising 12Val peptide, we pulsed autologous B-LCLs with the 12Val peptide and the 12Gly peptide (unmutated sequence) at 25 \( \mu \text{M} \), and performed a standard 4 h \(^{31}\text{Cr} \) release assay. As shown in Figure 3, the TLC 42-4 was capable of lysing autologous target cells when pulsed with the 12Val peptide, but when B-LCLs were pulsed with the control 12Gly peptide, no lysis was observed. Specific lysis was observed at all effector-target cell ratios tested. The cytotoxic effect of TLC 42-4 was not caused by lymphokine-activated killer (LAK) cell activity, since the natural killer (NK)LAK target K562 was not lysed (data not shown). Furthermore, cytotoxicity was blocked by anti-HLA DR MAbs, demonstrating that direct interaction between the TLC 42-4 and the autologous target cell was
expansion cells allow Here, it is being Cancer cell immunisation ras peptide homologous made mutated al.,1996). vaccination. peptides frequency sets Determination unsuccessful in mutated 12Gly peptide ( ) in 20 30 40 50 10 12Gly peptide expression 40-4 25 124-12Gly peptideexpressing 12Asp peptide compared with a T-cell precursor frequency of 1:110 000 PBMCs for the 12Gly peptide expressing normal ras (Figure 5). These results suggest that ras peptide immunisation of this donor may have produced a clonal expansion of at least two different sets of T cells, since the T-cell precursor frequencies are not identical.

Discussion

Cancer vaccines based on defined peptide epitopes are now being tested in a number of clinical trials. In these approaches it is of great importance to characterise the in vivo activated T cells of the responding patients after peptide vaccination. Here, we report that the induction of 12Val peptide-responsive T cells in the pancreatic carcinoma patient after mutant ras peptide vaccination resulted in a sufficient expansion of CD4+ T cells specific for the 12Val peptide to allow cloning of these T cells in vitro. Our vaccination strategy is based on earlier observation that ras peptide binding to HLA class II molecules is highly promiscuous (Geddé-Dahl et al., 1994; Johansen et al., 1994), indicating that a majority of cancer patients will carry one or more HLA class II molecules capable of binding and presenting the mutant ras peptide used for vaccination. Demonstrating of HLA-DR6 and DQ2 as ras peptide-presenting molecules in this patient supports this concept, since neither DR6 nor DQ2 have earlier been shown to bind or present ras peptides to T cells.

None of the five patients demonstrated a T-cell response towards the K-ras mutation found in their tumour before vaccination (Gjertsen et al., 1995). This could theoretically result from tolerance induction, leading to inactivation of T cells with the appropriate receptor. However, our results demonstrate that the lack of T-cell responsiveness observed initially in these patients was not due to absence of specific T cells in the repertoire or lack of HLA-molecules with
appropriate binding capacity, since a ras peptide-specific response could be induced upon vaccination. These results suggest that the state of unresponsiveness towards peptides expressing mutated K-ras epitopes may be overcome by peptide immunisation. The method of vaccination chosen in our approach is based upon loading of professional APCs with synthetic ras peptides ex vivo in order subsequently to present a tumour-specific epitope in an immunogenic context in vivo. Clearly, in two of our patients with terminal disease, including a large tumour burden, these peptide-loaded APCs were capable of initiating an immune response, and thus breaking a possible state of functional tolerance. The present approach is relatively crude, since we used freshly isolated, unfractionated PBMCs containing a small fraction of dendritic cells (DCs). Presumably, the peptide-loaded DCs are the active components of the vaccine. In future studies purified, freshly isolated or in vitro expanded DCs may prove more efficient. This last approach has recently been described for a MAGE-I peptide vaccination protocol, and was found to induce a T-cell response in vivo (Mukherji et al., 1995).

The clinical importance of the induced K-ras mutation-specific T cells remains to be established. Theoretically, CD4+ ras-specific T cells may influence the growth of the tumour in two different ways, either indirectly by recognising processed p21 ras protein taken up by professional APC residing in the tumour microenvironment, or directly by recognising a ras peptide presented by HLA class II molecules on the tumour cell itself. In the indirect mechanism, activation of the CD4+ T cell may result in the initiation of an effector cascade involving CD8+ effector T cells specific for a variety of tumour-specific or associated antigens. A number of such T-cell epitopes have now been defined for melanomas (Boon et al., 1994), but so far not for pancreatic adenocarcinomas. In the direct mechanism, the CD4+ T cells may kill the tumour cell following induction of HLA class II molecules on the tumour cells by cytokines released during an indirect immune recognition phase. In an attempt to study the potential functional role of the induced K-ras mutation-specific T cells, we performed some studies with peptide-pulsed B-LCLs as target cells. This approach was chosen, since we did not have cancer cells or cell lines from the patient available for functional studies. With these ‘surrogate’ tumour cell lines expressing the appropriate ras peptides, we were able to demonstrate that in vivo activated T cells were potent killer cells. Killing was specific, since target cells pulsed with the non-mutated peptide were not killed. This indicates that activated, K-ras mutation-specific T cells may have a direct functional importance as killer cells in vivo, even though they are of the T-helper phenotype (CD4+). Consistent with that contention, human CD4+ T-cell lines, generated by in vitro stimulation with mutant ras peptides, have been shown to be able efficiently to kill B-LCLs transduced with the corresponding p21 ras oncogene (Tsang et al., 1994). Future studies with HLA-matched tumour cell lines will hopefully allow a more detailed knowledge of the functional properties of these in vivo activated, cytotoxic T cells.

The difference between the mutant ras peptides and the peptide representing normal p21 ras is only one amino acid in position 12, and can give rise to T cells that may show varying degrees of cross-reactivity (Gedde-Dahl et al., 1992b). This presents the possibility that some T cells may be autoreactive and, therefore, potentially harmful. In our pilot clinical study (Gjertsen et al., 1996) this did not seem to be the case, since no side-effects or possible auto-reactivity were observed in the patient having cross-reactive T cells. In a mouse model system, where CD8+ T cells specific for different ras epitopes were induced following immunisation with a ras-vaccinia virus construct, only T cells specific for mutant ras were able to lyse target cells harbouring a ras mutation (Skipper et al., 1993), indicating that endogenous expression of p21 ras by normal cells may only result in subthreshold amounts of ras peptide and is therefore insufficient for T-cell recognition. In this context, it is of importance that in many cases of human malignancies, the ras oncogene family seems to be overexpressed compared with normal tissues (Slamon et al., 1984; Spandidos and Kerr, 1984). For the base-reactivity against the common mutation in position 12, as well as the peptide expressed normal ras observed in donor 2, seemed to be a result of in vivo activation of a set of T cells, which was cross-reactive. The precursor frequency of these cells (1:110 000) was lower than the precursor frequency of T cells recognising the Asp12 mutation. This precursor frequency may be too low to cause any side-effects in the form of autoimmunity.

In conclusion, we have shown that ras peptide vaccination of patients with pancreatic adenocarcinoma can result in the induction of T cells specific for combinations of ras peptides and HLA class II molecules not previously demonstrated. These T cells are functionally active and can kill autologous peptide-pulsed target cells specifically. Such T cells may be of clinical benefit to patients with minimal residual disease. Studies are currently under way to test this approach in patients with colorectal adenocarcinoma and pancreatic adenocarcinoma after surgery.

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