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**CD8** \(^+\) T cells from a patient with colon carcinoma, specific for a mutant p21-Ras-derived peptide (GLY\(^{13}\) → ASP), are cytotoxic towards a carcinoma cell line harbouring the same mutation

Received: 4 October 1994 / Accepted: 1 December 1994

**Abstract** Several T lymphocyte clones (TLC), specific for a p21-Ras-derived peptide expressing a Gly\(^{13}\) → Asp mutation and of the CD8\(^+\) subtype, were generated from peripheral blood of a colon carcinoma patient. The TLC exerted cytotoxicity against an interferon-\(\gamma\) (IFN\(\gamma\))-pretreated colon carcinoma cell line, HCT116, which harbours the Gly\(^{13}\) → Asp mutation and shares both HLA-A2 and HLA-B12(44) with the patient. This cytotoxic effect could be blocked by a monoclonal antibody (mAb) against CD8 molecules, as well as with a mAb against HLA class I molecules and a polyclonal antiserum against HLA-B12, identifying B12(44) as the antigen-presenting molecule. In growth-inhibition experiments, the growth of both IFN\(\gamma\)-pretreated and untreated target cells were strongly inhibited by the presence of the CD8\(^+\) TLC. Together these data indicate that human cancer cells harbouring a spontaneous ras mutation can process aberrant p21 Ras and express peptide/HLA-class-I complexes on their surface in sufficient density to be recognized by Ras-specific cytotoxic T lymphocytes.

**Key words** Mutant p21 Ras · Colon carcinoma · Cytotoxic T cells · HLA-B12(44)

**Introduction**

Mutations in the K-ras gene occur in 40%–50% of colon adenocarcinomas [19, 2, 3]. Aberrant p21 Ras, carrying an amino acid substitution as a result of a mutation, may serve as tumour-specific proteins and thus give rise to immunogenic peptides. Mutant p21 Ras or mutant p21-Ras-derived peptides have been shown to be immunogenic both in healthy individuals [14, 11, 12, 6] and in cancer patients [10, 7, 8]. In murine model systems, cytotoxic, MHC-class-I-restricted, Ras-specific T cells, have been described [16]. These T cells recognized two different epitopes, of which one encompassed a mutation in position 61. Cytotoxic T cells, specific for mutant p21 Ras, could also be elicited after immunization with mutant p21 Ras [5]. These T cells were capable of protecting immunized mice against syngeneic tumour cells carrying the corresponding mutation (Arg\(^{12}\)), but not against tumour cells carrying another mutation (Val\(^{12}\)). In these experiments, neither the effector cells nor the peptide epitopes recognized were further characterized. In man, cytotoxic T cells specific for mutant p21 Ras have so far not been described.

In a group of 251 patients with colorectal cancer, a Gly\(^{13}\) → Asp mutation was found to constitute 27% of the K-ras mutations detected [3]. In a previous report, we described CD4\(^+\) and CD8\(^+\) T cell clones (TLC) from a patient with a colorectal adenocarcinoma, which responded specifically to a synthetic peptide expressing this mutation, [\(^{13}\)Asp]1–25 [7]. The specificities of the p21 Ras Gly\(^{13}\) → Asp-specific CD4\(^+\) TLC from this patient described have been extensively characterized [8]. Here we have studied the functional aspects of some CD8\(^+\) TLC recognizing the Gly\(^{13}\) → Asp mutation from this patient and report that these T cells are capable of killing a colon adenocarcinoma cell line carrying the same mutation and sharing HLA class I molecules with the patient. We
also provide evidence that HLA-B12(44) is the antigen-presenting molecule.

Materials and methods

T cell donor

The female patient RM, 57 years old at the time of diagnosis, had a moderately differentiated adenocarcinoma, approximately 15 x 5 mm, located in the distal part of the rectum. Treatment included surgery, irradiation and cytotoxic treatment, but the patient died of the disease 4 years after the initial diagnosis. Screening of peripheral blood and subsequent generation of TLC were performed 2 years after diagnosis. The HLA type of the patient was HLA-A2, B12(44), 15(62); DR4,DRB1*0401,0402; DQ7,8 (DQA1*0301, DQB1*0301,0302).

Cells and media

Peripheral blood mononuclear cells (PBMC) were isolated after defibrination and centrifugation over Lymphoprep (Nycomed, Oslo, Norway). The human colon carcinoma cell line HCT116 (American Type Culture Collection, ATCC, Rockville, Md.) contains a Gly→Asp mutation in the K-ras oncogene as demonstrated by Jiang et al.[11]. This was confirmed in our laboratory by sequencing the ras allele. Serological (HLA class I typing [18] identified that HLA-A1, B12(44), 15(63) were expressed, while genomic typing [15] revealed that HCT116 carried the DQB1*0301 gene, i.e. DQ7.

A B-lymphoblastoid cell line (B-LCL) was generated by Epstein-Barr-virus (EBV) transformation of B cells from patient RM (RM-Eb). Homozygous B-LCL from the 10th and 11th International Histocompatibility Workshops (IHWS) cell panel included: 9009 (KAS011), 9031 (Boleth), 9052 (DBB) and 9054 (Ek). The HLA profiles of the different cell lines used are given in Table 1.

All cultures were grown in RPMI-1640 medium (Gibco, Paisley, UK) with gentamicin, 15% heat-inactivated human pool serum (T cells) or 10% fetal calf serum (Gibco) (cell lines).

Antibodies

mAb used included W6/32 (panreactive anti-HLA-class-I; from the 10th IHWS), 8G12 (anti-HLA-A2) [1], L243 (anti-HLA-DR; ATCC) and ITI-5C2 (anti-CD8) [9]. The human polyclonal antiserum ofL (anti-HLA-B12) was previously produced in our laboratory (Thorsby, unpublished).

Peptides

The unmutated K-ras-derived peptide 1–25 (Gly13), the mutated [13Asp]1–25 peptide and truncations of this, were synthesized as described earlier [11].

T cell clones (TLC)

The CD8+ TLC, specific for a p21 K-ras-derived peptide carrying a 13Gly→Asp mutation, were generated from patient RM as described earlier [7]. Briefly, PBMC were stimulated in vitro with the peptide [13Asp]1–25 at 16.5 µM in the presence of 5 U/ml recombinant interleukin-2 (rIL-2). At day 7, T cell blasts were cloned using allogeneic, irradiated (30 Gy) PBMC as feeder cells, 1 µg/ml phytohaemagglutinin (Wellcome, Dartford, UK) and 5 U/ml rIL-2 (Amersham, Aylesbury, UK). Growing TLC were propagated with allogeneic feeder cells, 1 µg/ml phytohaemagglutinin and 30 U/ml rIL-2.

Proliferative assays

The CD8+ TLC generated were screened for proliferative responses against the original stimulating [13Asp]1–25 peptide. Owing to a lack of autologous PBMC, a mixture of two HLA-class-I-matched B-LCL, Boleth (9031) and Ek (9054), was used as antigen-presenting cells; their HLA profiles are given in Table 1. A total of 7 x 104 antigen-presenting cells irradiated with 100 Gy, were pulsed with the [13Asp]1–25 peptide at 30 µM for 4 h before addition of (2–3)x104 T cells. rIL-2 was added at 2.5 µg/ml, where indicated, and proliferation was measured at day 3 after coinubation with 1 µCi [3H]thymidine (Amersham) 18 h prior to harvesting.

Cytotoxicity assays

Cytotoxicity of CD8+ TLC against the colon carcinoma cell line HCT116, or control cell lines, was measured in a 4 h 51Cr-release assay. Target cells, pretreated with IFNγ (Genzyme, Cambridge, Mass.) for a minimum of 3 days, were incubated with 7.5 MBq 51Cr and fetal calf serum 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 at 2 x 103 cells/well in round-bottomed 96-well plates (Costar, Cambridge, Mass.). Effector cells were added in different numbers as indicated. In antibody-blocking experiments, target cells were incubated with mAb for 1 h at 37°C prior to addition of T cells. Controls included target cells alone with mAb at the highest concentration to exclude unspecific effects mediated by the mAb (data not shown). In assays with peptide-pulsed target cells, autologous B-LCL were incubated with the different peptides for 1 h at room temperature before addition of T cells. 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). The percentage specific chromium release was calculated by the formula: 100 x (experimental release – spontaneous release)/(maximum release – spontaneous release).

Growth-inhibition assays

HCT116 cells, pretreated with IFNγ (100 U/ml) where indicated, were plated at 2 x 103 cells/well in round-bottomed 96-well plates. Proliferation was measured at day 2, after addition of 1 µCi [3H]thymidine 18 h prior to harvesting (Fig. 4A, B). T cells were not irradiated, as these cells did not proliferate upon stimulation with HCT116 cells (data not shown). In antibody-blocking experiments, mAb at the given concentrations were preincubated with target cells (pretreated with 1000 U/ml IFNγ) 30 min prior to addition of the TLC, and [3H]thymidine incorporation was measured at day 4 (Fig. 4C).

Results

In PBMC from a colon carcinoma patient, RM, a IL-2-dependent T cell response was detected in vitro at day 7 against a mutant-Ras-derived peptide carrying a 13Gly→Asp mutation as described earlier [7].