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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

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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-γ (IFNγ)-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 IFNγ-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)

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Introduction

Mutations in the K-*ras* gene occur in 40%–50% of colonic 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 colonic 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 15×5 mm, located in the distal part of the rectum. Treatment included surgery, irradiation and cytostatic 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 $Gly^{13} \rightarrow Asp$ mutation in the K-*ras* oncogene as demonstrated by Jiang et al. [13]. This was confirmed in our laboratory by sequencing the mutant *ras* allele. Serological (HLA class I) typing [18] identified that HLA-A1,2; 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 ØL (anti-HLA-B12) was previously produced in our laboratory (Thorsby, unpublished).

Peptides

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

T cell clones (TLC)

$CD8^+$ TLC, specific for a p21 K-*ras*-derived peptide carrying a $^{13}Gly \rightarrow Asp$ mutation, were generated from patient RM as described earlier [7]. Briefly, PBMC were stimulated in vitro with the peptide [^{13}Asp]1–25 at 16.5 μM in the presence of 5 U/ml recombinent 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 [^{13}Asp]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 7×10^4 antigen-presenting cells irradiated with 100 Gy, were pulsed with the [^{13}Asp]1–25 peptide at 30 μM for 4 h before addition of $(2–3) \times 10^4$ T cells. rIL-2 was added at 2.5 U/ml, where indicated, and proliferation was measured at day 3 after coincubation with 1 μCi [^{3}H]thymidine (Amersham) 18 h prior to harvesting.

Cytotoxicity assays

Cytotoxicity of $CD8^+$ TLC against the colon carcinoma cell line HC116, or control cell lines, was measured in a 4 h ^{51}Cr-release assay. Target cells, pretreated with IFNγ (Genzyme, Cambridge, Mass.) for a minimum of 3 days, were incubated with 7.5 MBq ^{51}Cr 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 2×10^3 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 ^{51}Cr 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: $100 \times$ (experimental release − spontaneous release)/(maximum release − spontaneous release).

Growth-inhibition assays

HCT116 cells, pretreated with IFNγ (100 U/ml) where indicated, were plated at 2×10^3 cells/well in round-bottomed 96-well plates. Proliferation was measured at day 2, after addition of 1 μCi[^{3}H]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 [^{3}H]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 $Gly^{13} \rightarrow Asp$ mutation as described earlier [7]. The

responding T cells were cloned at day 7 and several TLC of both the $CD4^+$ and $CD8^+$ phenotype were generated. In the initial screening, and in experiments performed shortly after generation of the $CD8^+$ TLC, peptide-specific, proliferative responses to the $Gly^{13} \rightarrow Asp$ peptide were obtained [7]. However, the proliferative response was lost upon propagation of the TLC in vitro, but they still proliferated in response to IL-2 (data not shown). In cytotoxicity tests we used an established colon carcinoma cell line, HCT116, expressing the $Gly^{13} \rightarrow Asp$ mutation as target cells, since it was not possible to obtain a biopsy or cancer cell line from patient RM. Serological and genomic typing revealed that this cell line shares several HLA alleles with the patient (Table 1). However, only HLA class I molecules were expressed on the surface of HCT116, and it was not possible to induce expression of HLA class II molecules by treatment with IFNγ.

Table 1 HLA types of cell lines used in proliferative and cytotoxicity assays. *ND* not determined

Cell line	HLA-A	HLA-B	HLA-DQ	HLA-DR
RM Eb	2	12(44), 15(62)	7, 8	4
HCT116	1, 2	12(44), 15(63)	7[a]	ND
9031	2	15(62)	8	4
9054	2	12(44)	5	14
9009	1	37	1	2
9052	2	57	9	7

[a] Genomic typing (DQB1*0301), HCT116 does not express HLA class II molecules

A colon carcinoma cell line, HCT116, harbouring the $Gly^{13} \rightarrow Asp$ mutation, is killed by $Gly^{13} \rightarrow Asp$-peptide-specific $CD8^+$ TLC

Three $CD8^+$ TLC (RM4, RM35 and RM57) were selected for further studies based on their proliferative responses observed in the initial screening [7]. All three TLC were found to exert cytotoxicity against the target cell line HCT116 (Fig. 1). The cell line had to be treated with IFNγ in order to be lysed efficiently. RM4 and RM57, however, required a high number of T cells to exceed 20% killing (effector : target ratio of 50 : 1). This was not due to insufficient amounts of IFNγ, since the cytotoxic capacities of these TLC could not be enhanced by increasing the amount of IFNγ used in pretreatment of the target cell line (data not shown). IFNγ treatment had no effect on the overall expression of HLA class I molecules or adhesion molecules (ICAM, 1, 2, 3, B7) on HCT116 (flow cytometry, data not shown). The most potent TLC, RM35, was chosen for further studies.

In control experiments, we showed that TLC RM35 did not kill EBV-transformed B cells expressing HLA-A1 (target 9009), which is an HLA-A molecule carried by HCT116 and not by the patient, demonstrating that the cytotoxicity against HCT116 was not due to alloreactivity against HLA-A1. In addition, neither autologous EBV-transformed B cells (RM-Eb) nor an unconnected B-LCL (9052) were killed (Table 2), supporting the suggestion that RM35 specifically recognizes an antigen presented by the target cell line HCT116.

In order to confirm that killing of the HCT116 cell line carrying the Asp^{13} mutation correlates with the

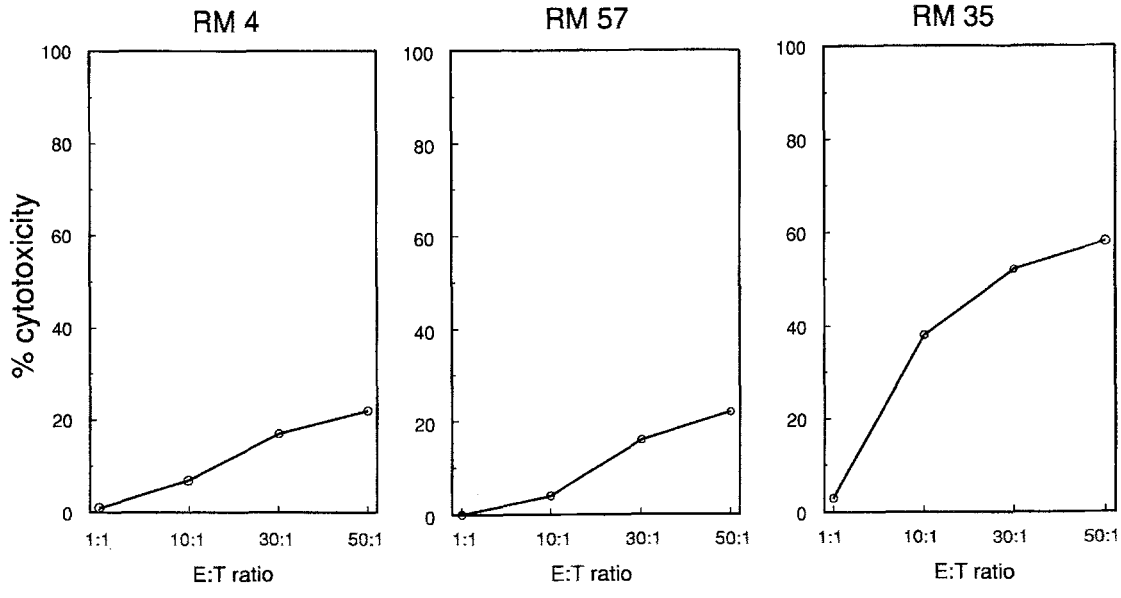

Fig. 1 Cytotoxicity of three $CD8^+$ T lymphocyte clones (TLC) from patient RM (RM4, RM57 and RM35) against the colon carcinoma cell line HCT116. HCT116 was pretreated with interferon γ (IFNγ) at 100 U/ml and 1000 U/ml in the assay with RM4/RM57 and RM35 respectively. Spontaneous ^{51}Cr release was below 15%. Standard deviation of triplicates was usually below 10%

Table 2 Cytotoxicity of the $CD8^+$ T lymphocyte clone RM35 against different target cell lines. The HLA profiles of the target cell lines are given in Table 1

Target	Cytotoxicity (%) at an E:T ratio of			
	1:1	10:1	30:1	50:1
HCT116	3.0	28.7	43.4	41.8
9009	< 0	1.0	< 0	1.0
9052	1.2	5.3	3.5	4.1
RM-Eb	< 0[a]	1.0	1.0	< 0

[a] Standard deviation of triplicates was usually below 10%

peptide-specific proliferative capacity of the TLC seen during the early screening, we compared the cytotoxic capacity of RM35 with that of two other $CD8^+$ TLC generated in the same cloning experiment but lacking peptide specificity. Only the TLC that initially was able to mount a peptide-induced IL-2-dependent proliferative response also lysed HCT116 (Fig. 2), demonstrating that the observed killing is associated with the peptide specificity of the TLC.

Identification of HLA-B12(44) as the antigen-presenting molecule

Since the target cell line HCT116 did not express HLA class II molecules, and HLA-A2 and B12(44) were shared with the patient, the latter molecules were candidate antigen-presenting HLA molecules. First we performed some blocking experiments with mAb directed against HLA or CD8 molecules. The results illustrated in Fig. 3A demonstrate that the cytotoxic effect of RM35 on the target cell line HCT116 was completely inhibited in the presence of a panreactive HLA-class-I mAb (W6/32), but remained unaltered or was enhanced in the presence of an anti-HLA-A2 mAb (8G12) or a mAb against HLA class II (L243, anti-HLA-DR). A monoclonal antibody against B12 was not available to us. We therefore instead used a human polyclonal antiserum against HLA-B12 (serum ØL), recognizing both splits of the B12 molecule (44 and 45). The concentration of anti-B12 in this serum is low compared to that of monoclonal antibodies. Results shown in Fig. 3B show that this antibody nevertheless inhibits cytotoxicity in a concentration-dependent manner (82.8% inhibition in a 1:2 dilution). Together these results suggest that HLA-B12(44) is the antigen-presenting molecule. Also a mAb against CD8 (ITI-5C2) strongly blocked the response (Fig. 3C).

The effect of RM35 on growth of HCT116 in vitro

In addition to direct lysis of the target cells, $CD8^+$ effector T cells may affect the growth of target cells via

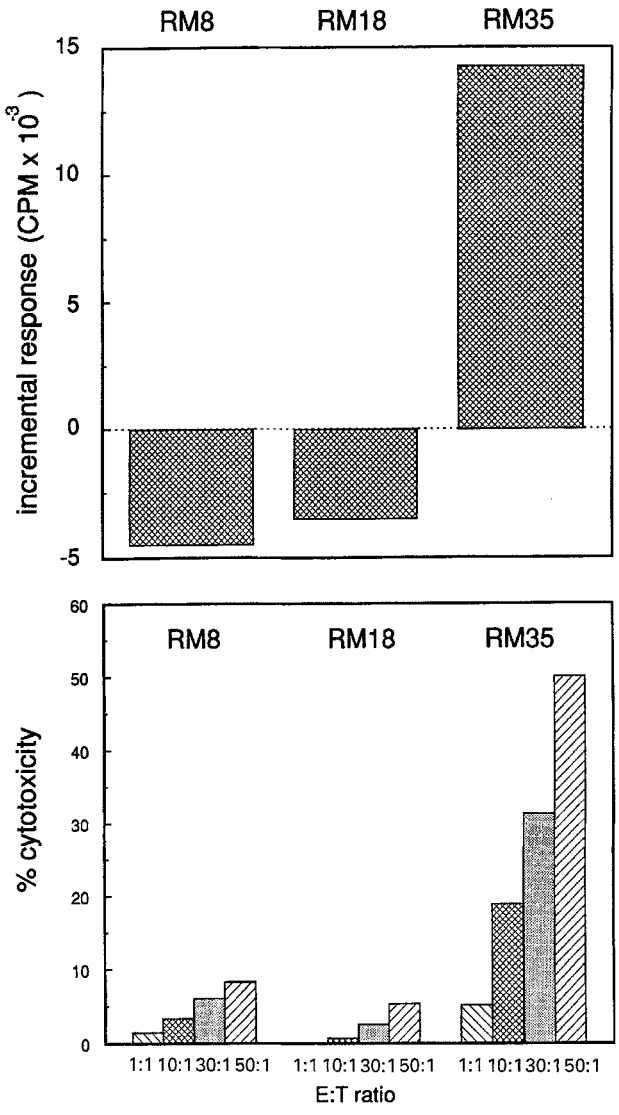

Fig. 2 Proliferative and cytotoxic capacities of three different $CD8^+$ TLC (RM8, RM18, RM35). *Upper panel* Proliferation was measured against [Asp^{13}] 1–25-peptide-pulsed HLA-class-I-matched B-lymphoblastoid cells (B-LCL), as described in Materials and methods. Values are given as incremental responses [(TLC + antigen-presenting cells + peptide + rIL − 2) − (TLC + rIL − 2)]. Background values (TLC + rIL-2) were 42279 ± 1952, 46141 ± 585 and 10072 ± 882 cpm for RM8, RM18 and RM35 respectively. Except for RM18 (SD = 13.2%), SD were below 10%. No peptide-specific proliferative responses were observed in the absence of recombinant interleukin-2 (rIL-2) (TLC + antigen-presenting cells + peptide). *Lower panel.* The cytotoxic capacities of TLC RM8, RM18 and RM35 against the ^{51}Cr-labelled target cell line HCT116, expressing the Asp^{13} mutation, at E:T ratios indicated. Spontaneous ^{51}Cr release was 11%, and SD of triplicates was usually less than 10%

other mechanisms. To assess the efficacy with which RM35 might influence the growth of HCT116 in vitro over longer periods, growth-inhibition assays were performed. Proliferation of HCT116, both untreated and pretreated with IFNγ, was measured in the presence or absence of RM35 at day 2 of coculture. Interestingly, in these experiments, RM35 profoundly inhibited the growth of both untreated HCT116 cells (Fig. 4A) as

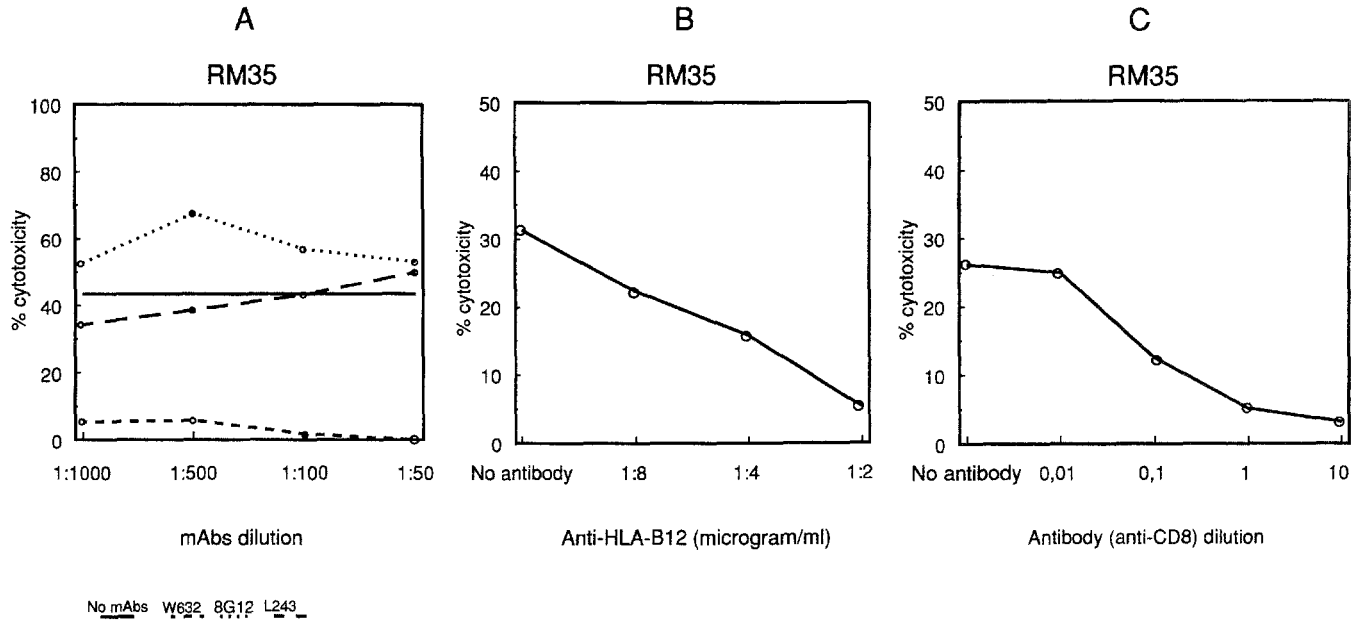

Fig. 3 **A** Inhibition of the cytotoxic effect of TLC RM35 against the IFNγ (1000 U/ml)-pretreated colon cancer cell line HCT116 by mAb against various HLA molecules: W6/32 (panreactive anti-class-I), 8G12 (anti-A2) and L243 (panreactive anti-DR). The mAb were used as dilutions of ascites as indicated. Spontaneous ^{51}Cr release was below 10%. SD of triplicates was usually below 10%. **B** Inhibition of the cytotoxic effect of RM35 against IFNγ-pretreated HCT116 (500 U/ml) with the human polyclonal antiserum ØL (anti-HLA-B12; recognizing both splits B-44 and -45). The antibody was used in twofold dilutions of serum, and the E:T ratio was 30:1 Spontaneous ^{51}Cr release was 11%, and SD of triplicates was below 10%. **C** Inhibition of the cytotoxic effect of RM35 against the IFNγ-pretreated HCT116 cell line (100 U/ml) with mAb against the CD8 molecule, ITI-5C2. The mAb was added at tenfold dilutions of ascites, and the assay was performed with an E:T ratio of 30:1. Spontaneous ^{51}Cr release was below 10% and SD of triplicates was usually less than 10%

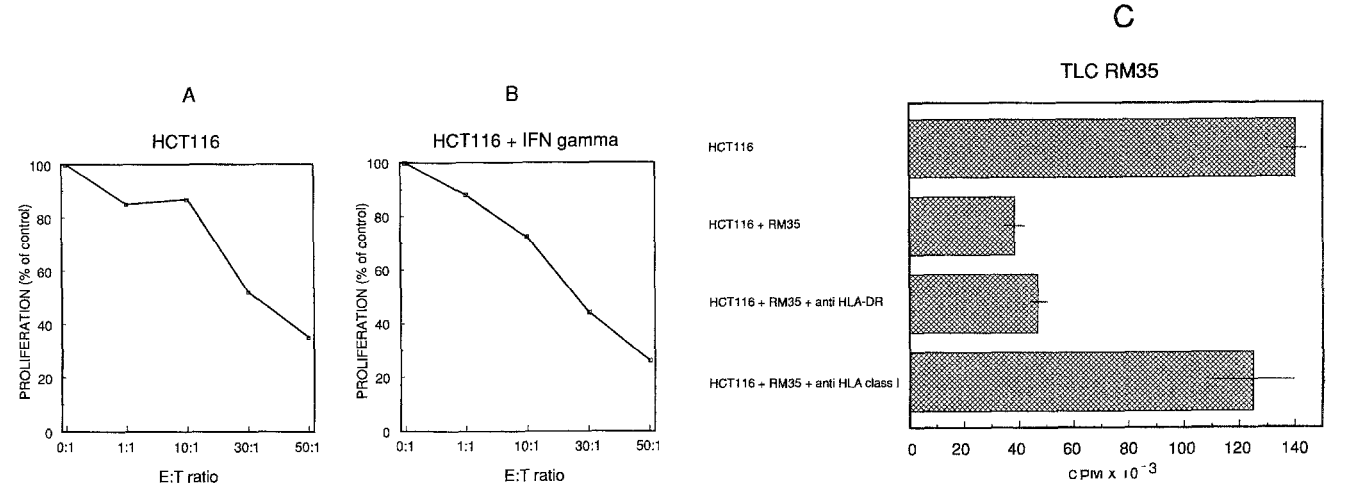

Fig. 4A, B Growth inhibition of the cell line HCT116 in the presence of RM35 measured as [^{3}H]thymidine incorporation at day 2. Inhibition was measured against untreated (**A**) or IFNγ-pretreated (100 U/ml) (**B**) target cells, at different E:T ratios as indicated. Background proliferation of target cells alone was 18180 ± 521cpm (**A**) and 14510 ± 3511 cpm (**B**). SD of remaining triplicates was usually below 10%. **C** The effect of mAb against HLA class I and II molecules on the growth-inhibitory capacity of RM35 on the cancer cell line HCT116. HCT116 were pretreated with 1000 U/ml IFNγ and seeded at 2×10^3 cells/well. mAb against HLA class I (W6/32) and HLA-DR (L243) were used at final concentrations of 1:50 diluted ascites and 25 μg/ml respectively. T cells were added at 60×10^3 cells/well. Proliferation was measured at day 4, after overnight incubation with 1 μCi[^{3}H]thymidine

well as HCT116 cells pretreated with IFNγ (Fig. 4B). In subsequent experiments we could show that the inhibitory effect of RM35 slightly increased when the assays were performed for 3 or 4 days, with a maximum inhibition of IFNγ-pretreated target cells at day 4 (82.5% inhibition with an E:T ratio of 50:1, not shown). Also in these experiments, the effect of RM35 on HCT116 cells was dependent on HLA class

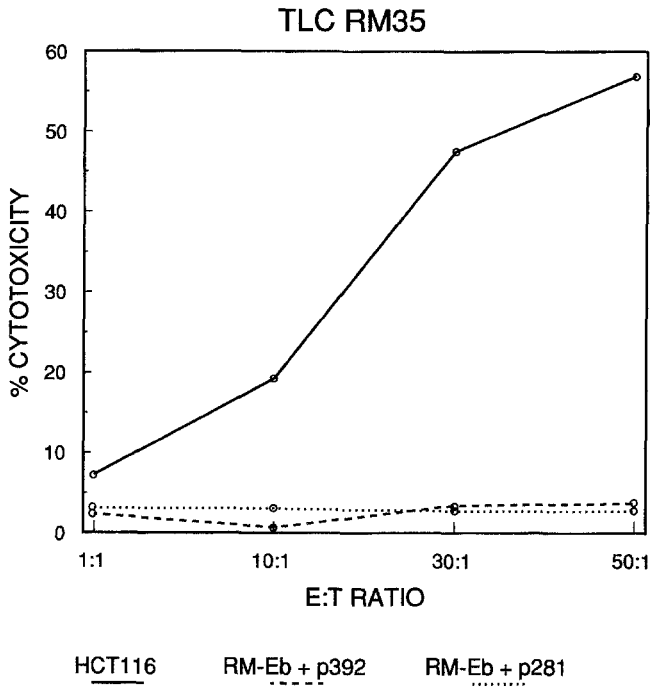

Fig. 5. Cytotoxicity of RM35 against HCT116 cells pretreated with 500 U/ml IFNγ, autologous Epstein-Barr-virus-transformed B cells (*RM-Eb*), pretreated with 500 U/ml IFNγ and pulsed with peptide 392 (GDVGKSALTI) or peptide 281 (GDVGKSALT) at 10 μM. Spontaneous ^{51}Cr release was below 20%, and SD of triplicates was usually less than 10%

I molecules, as mAb against HLA class I almost abolished the growth-inhibitory effects of RM35, while an anti-DR antiserum had no effects (Fig. 4C).

Attempts to identify the peptide epitope recognized by TLC RM35

Having identified HLA-B12(44) as the antigen-presenting molecule, we attempted to define the peptide epitope recognized by RM35, by loading autologous EBV-transformed B cells with mutant peptides encompassing the mutation. Recently a peptide binding motif for B44 has been identified (Coligan, personal communication). On the basis of these data, two likely candidate peptides (one 9-mer and one 10-mer) were tested. Killing of HCT116 was used as a control. Results shown in Fig. 5 demonstrate that the EBV-transformed target cells preincubated with the candidate peptides were not killed by RM35. These experiments were repeated several times with different peptide concentrations, always with negative results. Thus, with this approach we were unable to identify the peptide epitope being recognized by TLC RM35.

Discussion

Cytotoxic T cells, specific for aberrant gene products of cancer cells, are of great potential importance in cancer immunotherapy. Here, we report that $CD8^+$ TLC, specific for a $Gly^{13} \rightarrow Asp$ mutation of p21 Ras and generated from a colon cancer patient, are capable of killing a colon cancer cell line harbouring the corresponding K-*ras* mutation and sharing several HLA class I molecules with the patient. For one of these TLC, we demonstrate that one of the shared HLA molecules, B12(44), is the antigen-presenting molecule. Together, these results provide evidence for endogenous processing of mutant p21 Ras, to yield a peptide fragment that is subsequently presented to the $CD8^+$ T cell by a HLA-B12(44) molecule, and show for the first time that human cancer cells harbouring a naturally occurring *ras* mutation can be killed by *ras*-specific CTL (cytotoxic T lymphocytes).

The precursor frequency of Asp^{13}-reactive T cells in the colon carcinoma patient described, was sufficiently high to allow a detectable proliferative response in vitro at day 7, indicating a prior clonal expansion resulting from in vivo exposure to this mutation.

This IL-2-dependent, peptide-specific proliferative response was only seen in peripheral blood and in cloned $CD8^+$ T cells at early stages after their generation. Subsequent in vitro propagation of the TLC using phytohaemagglutinin in the presence of IL-2 and allogeneic feeder cells, resulted in loss of Ras-peptide-specific, proliferative responses. Unresponsiveness to the HCT116 cell line harbouring the Asp^{13} Ras mutation was observed at this stage, although the TLC now responded well to IL-2. These results indicate that the IL-2 receptors of the $CD8^+$ clones at an early stage could be up-regulated by recognition of a specific antigen, and that IL-2 receptors later on were constitutively expressed.

The cytotoxic and growth-inhibitory effect of $Gly^{13} \rightarrow Asp$-specific, HLA-B12(44)-restricted $CD8^+$ TLC described here, together with our previous findings of HLA-DQ7-restricted $CD4^+$ T cells of the same specificity in this patient, might indicate that a coordinated response against the mutant Ras protein has taken place, resulting in eradication of tumour cells harbouring the mutation. This might explain why no Ras mutation was detected in tumour cells from the patient, as discussed earlier [7, 8].

For efficient lysis of HCT116 by the Asp^{13}-specific CTL in short-term assays, pretreatment of the target cell line with IFNγ was necessary. Similar observations have been made for HLA-B-restricted CTL in a melanoma system [20] and for autologous HLA-Bw6-restricted CTL clones specific for human pancreatic carcinoma [21]. The enhancing effect of IFNγ on the susceptibility of tumour cells to lysis has been explained by the effect of expression of HLA class I and adhesion molecules such as ICAM-1 [17]. Since we observed no enhanced expression of these molecules after treatment, additional IFNγ effects have to be considered, for instance increased processing of mutant p21 Ras. In this context, it is of interest that

proteasomes, including MHC-encoded subunits, are regulated by IFNγ [23]. IFNγ was not required for growth inhibition of HCT116 exerted by RM35. Since it is known that IFNγ can be produced by TLC themselves upon recognition of target cells, in these experiments sufficient amounts of IFNγ may have been produced by the TLC during coculture. Alternatively, the mechanisms for growth inhibition may be independent of the effects of IFNγ.

Experiments to define the peptide epitope by loading autologous EBV-transformed B cells with synthetic peptides with the putative B44 binding motif encompassing the mutation have so far failed. We do not know the reason for this, but our results are reminiscent of those recently published by Wölfel et al. [22]. This experiment was directed at defining an antigen recognized by an HLA-A2-restricted, melanoma-specific CTL. Evidently, endogenously processed peptides were well recognized, resulting in killing of the melanoma cell line. Killing was also observed after transfection of the gene encoding tyrosinase or fragments of this gene. On the other hand, loading HLA-A2 target cells with the synthetic tyrosinase peptide representing the HLA-A2 binding motif, failed to induce significant killing unless very high peptide concentrations were used and/or the cells were pretreated with an A2 antibody that enhanced, rather than blocked, antigen presentation. At present no such antibody against HLA-B44 is available for testing in our system.

These findings may be explained in several ways. T cell killing is critically dependent on the epitope density on the target cell. For some antigens, endogenous loading of HLA class I molecules may be more efficient and result in higher antigen density on the cell surface than when peptide is provided from the outside. The epitope density may be of particular relevance when TLC with low-affinity receptors are involved.

Alternatively, the short peptides added to the cells in culture may be degraded by proteolytic activity in the serum [4] or by peptidases such as aminopeptidase located in the cell membrane (Buus, personal communication). Such enzymes may remove critical amino acids and thereby interfere with subsequent binding to HLA molecules. It is therefore conceivable that, in our experiments, the relevant Ras peptide, when added exogenously, might be destroyed before binding.

Our report demonstrate specific killing and growth inhibition of human colon carcinoma cells carrying a defined *ras* mutation by HLA-class-I-restricted $CD8^+$ TLC. The TLC used were generated from a colon cancer patient by stimulation of PBMC in vitro with synthetic peptides corresponding to the same mutation. The results reported make it very likely that recognition is due to presentation of endogenously synthesized mutant Ras by HLA B44 molecules present on the target cell, although this could not be proven by the use of synthetic peptides.

Generation of effector T cells recognizing tumor-specific antigens such as mutant-Ras-derived peptides, and defining key elements such as the antigen-presenting molecules and peptide composition, constitute the basis for a rational immunotherapeutic approach to treatment of human cancers harbouring *Ras* mutations.

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