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In vitro generation of cytotoxic T lymphocytes against mutated *ras* peptides

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Mutations of the ras proto-oncogenes represent frequent genetic alterations in human cancers. These mutations appear as single-point mutations causing single amino acid substitutions at residues 12, 13 or 61 (activated p21 ras proteins). Therefore, peptides encompassing ras mutations appear to represent an appealing target for active immunotherapy procedures. By using a computer program, selecting appropriate HLA-A2.1 binding motifs from defined protein sequences, ras nonapeptides encompassing mutations in positions 12 and 61 with a putative binding capacity for HLA-A2.1 molecules were identified and synthesized. Generation of primary cytotoxic T cell lymphocyte (CTL) response was attempted by weekly restimulations of peripheral blood lymphocytes from healthy donors in the presence of irradiated autologous Epstein Barr virus transformed lymphoblastoid cells (EBV cell lines) and IL-2 and IL-4. Periodically, cultured cells were tested for their killing capacity using as target cells HLA-A2.1+ EBV cells previously pulsed with different combinations of the peptides under investigation. After eight rounds of restimulation reproducible cytotoxic activity against EBV target cells pulsed with two nonamers encompassing ras 61 Gln→Leu mutation was detectable in one donor. Thus, the results obtained indicate that it is possible to induce from PBMC of healthy donors CTL specific for peptides encompassing 61 GLN→LEU ras gene mutation following repeated weekly in vitro restimulations.

Key words: oncogene protein p21 (ras); piont mutation; T-lymphocytes, cytotoxic

Introduction

Neoplastic transformation is caused by a stepwise accumulation of a series of genetic alterations affecting oncogenes and tumor supressor genes. The expression of mutated proteins encoded by these genes is restricted to abnormal cells thus raising the possibility to consider these molecules as tumor specific antigens. On the other hand, the long-standing goal of cancer immunotherapy is to stimulate the immune rejection of tumors. Based on the assumption that T lymphocytes might be able to eradicate cancer cells as effectively as they kill autologous virus-infected cells or allogeneic cells, tumor immunologists have

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been trying to identify specific target antigens displayed by cancer cells that could make them recognizable to cytolytic T lymphocytes (CTL). The identification and selection of these potential human cancer antigens and specific epitopes as targets for CTL is now in a highly dynamic phase. Specific peptides that bind to human major histocompatibility complex (HLA) molecules have now been identified for melanoma-associated antigens. The identification of other human carcinoma-associated antigens and epitopes that can be recognized by human T cells is also under active investigation. Molecules, such as prostate specific antigen (PSA), *c-erbB/2*, *MUC-1*, point mutated *ras*, point mutated *p53*, and carcinoembryonic antigen (CEA) are possible such candidates.[1,2,3]

We investigated the potential antigenic epitopes encompassing mutations of *ras* proteins using as responders, peripheral blood lymphocytes from healthy donors. Mutations of the *ras* proto-oncogenes represent frequent genetic alterations in human cancers, detectable in 90 % of pancreatic carcinomas, 40-50 % of colorectal tumors, 20-40 % of lung carcinomas and approximately 30 % of acute myelogenous leukemias. These mutations appear as single-point mutations causing single amino acid substitutions at residues 12, 13 or 61 (activated p21 *ras* proteins).[4-6]

Therefore, we explored the possibility to define conditions permitting the use of synthetic peptides encompassing „mutant" residues of activated *ras* proteins for the induction of specific CTL responses. In the present study we applied a recently described protocol,[7] which is based on stimulation of large numbers of naive lymphocytes with the antigen (peptides) and on concomitant application of Tetanus toxoid, IL-2 and of IL-4.

Materials and methods

Cells and media

The medium used throughout this study was RPMI 1640 supplemented with 2 mM L-glutamine, 1 % non-essential amino acids, 1 % sodium piruvate, penicillin (100 U/ml), streptomycin (100 mg/ml) (all from Gibco Ltd, Paisley, UK) and with 10 % heat inactivated human AB-serum (Blutspendezentrum, SRK, Basel) (complete medium). Human peripheral blood mononuclear cells (PBMC) were obtained from the heparinized peripheral venous blood from a group of HLA-A2.1 healthy donors. Mononuclear cells were isolated by standard gradient centrifugation over Lymphoprep cushion (800 g for 20 minutes). After two washings cells were resuspended in complete medium. PBMC were also used as a cellular source for the generation of Epstein-Barr virus (EBV) transformed lymphoblastoid cell lines.[8]

Synthetic peptides

Peptides were synthesized by solid-phase method using 9050 Millipore peptide synthesizer (Millipore, Volketswil, Switzerland). Synthesis was performed as suggested by the manufacturer. The peptides were purified to homogeneity and analyzed by HPLC. Their purity as analyzed by HPLC was routinely found to exceed 90 %. Synthesized peptides were designed by using a computer program (a gift of Dr. J. D'Amaro, Leiden, Holland;[9]) based on the amino acid motifs found in the known HLA-A2.1 binding peptides. This program represents a useful scanning tool for the identification of potential HLA-A2.1 restricted peptides. We thus synthesized a panel of seven peptide nonamers encompassing *ras* mutations in positions 12 and 61. The sequences of these seven nonamers (single-letter code sequences) together with their arbitrary HLA-A2.1 binding scores are

Table 1. Synthetic peptides used *in vitro* either for the generation of *ras* specific CTL or for the pulsing of EBV target cells in cytotoxic assays. An amino acid (a.a.) sequence and HLA-A2.1 binding score of the seven synthesized nonapeptides encompassing *ras* oncogene mutations in positions 12 and 61 is indicated

Peptide	Mutation		Amino acid sequence	HLA-A2.1
	a.a. position	a.a. change		binding score[x]
pool[x] 1				
ras 1	61	Q to L	$-G_{60}LEEYSAMR_{68}-$	96
ras 2	61	Q to L	$-L_{53}DILDTAGL_{61}-$	48
ras 3	61	Q to L	$-I_{55}LDTAGLEE_{63}-$	48
pool 2				
ras 4	61	Q to K	$-T_{58}AGKEEYSA_{66}-$	96
ras 5	61	Q to K	$-I_{55}LDTAKKEE_{63}-$	48
pool 3				
ras 6	12	G to V	$-V_{9}GAVGVGKS_{17}-$	128
ras 7	12	G to V	$-Y_{4}KLVVVGAV_{12}-$	96

[x] Initially, *in vitro* stimulation of PBMC was attempted by using pools of two or three synthesized peptides. Binding score, expressed as arbitrary units, was calculated by taking advantage of a specific computer program (9).

reported in Table 1. A correlation can be expected between high score and binding to HLA-A2.1.[9] The peptides were dissolved in DMSO (Sigma, Buchs, Switzerland) as a stock solution (at 20 mg/ml). For *in vitro* experiments they were further diluted in complete medium (10 mg/ml final concentration).

Generation of CTL.

Generation of primary CTL response was attempted by taking advantage of a recently described protocol.[7] The initial *in vitro* stimulations were performed by using „bulk cultures" containing large numbers of PBMC in the presence of synthetic peptides. During the initial seven days the medium was also supplemented with tetanus toxoid (1 mg/ml) to provide „helper" function. Briefly, 25-75 x 10^6 PBMC were stimulated with mixtures of ras-derived peptides (pool 1, pool 2 or pool 3 peptides, Table 1) in 10 ml of complete medium in the presence of tetanus toxoid (1 mg/ml). Subsequently, Ficoll purified lymphoblasts underwent weekly restimulations in the presence of irradiated, autologous EBV cells pulsed with combinations of appropriate peptides (pool 1, pool 2 or pool 3 peptides). Exogenous IL-2 and IL-4 were added twice per week at low doses (10 U/ml and 1 U/ml, respectively). Periodically, ^{51}Cr release tests (killing assays) were performed, by using, as target cells HLA-A2.1$^+$ EBV cells,

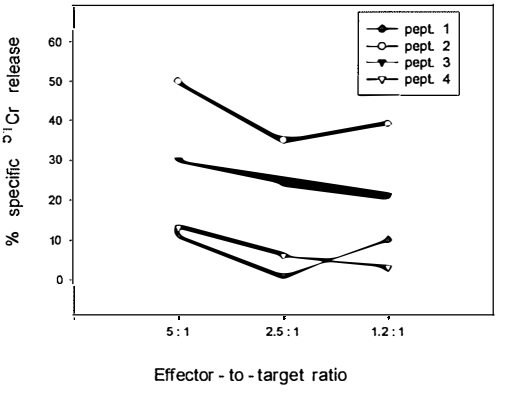

Figure 1. Specificity of bulk-cultured CTL undergoing in vitro re-stimulations with mutant *ras* peptides 1, 2 and 3 (pool 1 peptides). As target cells, HLA-A2.1$^+$ EBV-transformed B cells, pulsed with indicated individual peptides, were used.

previously pulsed with the different combinations of peptides under investigation. Briefly, EBV cells were first labelled with a $Na^{51}Cr$ solution (Dupont, Regensdorf, Switzerland) as described.[10] Afterwards, they were pulsed for 1 hr at 37°C with defined peptides at 10 mg/ml final concentration. Following extensive washings, target cells were resuspended at 10^5 cells/ml final concentration and added to different numbers of effector cells in U-bottom, 96-well trays. Cultures were incubated for 4 hours at 37°C in a 5 % CO_2 humidified atmosphere. Supernatants were then collected and specific ^{51}Cr release was calculated according to the standard formula as described.[10] Antibody blocking studies were performed by adding monoclonal antibodies in the form of hybridoma supernatants to killing assays, at 1:3 final dilution.

Results and discussion

Using a panel of mutated p21 *ras* peptides a significant specific killing could be detected after about 8 restimulation cycles in one PBMC donor out of five tested. We were able to demonstrate that the CTL recognized a p21 *ras* nonapeptide encompassing a Q→L (Gln→Leu) mutation in position 61. CTL recognized EBV target cells preincubated with either peptide 2 or peptide 3. The results from one such representative experiment are presented in Figure 1. Preincubation of EBV target cells with wild type peptides did not result in their recognition by CTL and subsequent killing (Table 2). Killing of EBV target cells preincubated with a mutant *ras* nonapeptide could be blocked by anti-HLA-A2.1 monoclonal antibodies, consisted with a specifically restricted recognition (Table 3). When further analyzed, our CTL appeared to be monoclonal in nature, since all $CD8^+$ T cell clones generated express Vb14 gene product in combination with Jβ2.7 and Cβ2 (data not shown,[11]).

Our data are in agreement with similar results obtained by others, by taking advantage of different culture conditions.[12] The characterization of mechanisms underlying HLA class I restricted antigen presentation and the identification of the peptide motifs allowing binding of antigenic epitopes to defined HLA determinants permit testing the immunogenicity of specific reagents in terms of capacity to induce CTL responses. Considering the role of mutated oncogenes or of tumor suppressor genes in the transformation processes and their specific expression in neoplastic cells, their products could represent the ultimate target for tumor specific active immunotherapies. Accordingly, we report here that we were able to generate *in vitro a* primary peptide specific CTL response

Table 2. Recognition of EBV target cells pulsed by wild type *ras* peptide by CTL generated with the pool 1 of mutated *ras* peptides

Effector to target ratio	% specific ^{51}Cr release EBV target cells preincubated with	
exp. 1	mutant *ras* 2 peptide	wild type peptide
2:1	32	0
1:1	28	1
0.5:1	12	0
exp. 2	mutant *ras* 3 peptide	wild type peptide
5:1	56	13
2.5:1	35	2
1.2:1	22	0

Table 3. Effect of anti-HLA-A2.1 monoclonal antibody on the killing of EBV target cells

Effector to target ratio	% specific ^{51}Cr release EBV target cells preincubated with *ras* 3 mutant peptide monoclonal antibody	monoclonal antibody
	anti-HLA-A2.1	anti-Mage3
10:1	7	69
5:1	5	72
2.5:1	1	45
1.2:1	3	32

against peptides encompassing mutation at position 61 in *ras* oncogene. The limitations of the experimental system described above are the number of restimulation cycles (8 rounds) and low frequency of responding donors (one out of five). Therefore, research for more practical immunization conditions is currently being pursued. In addition, the capacity to generate specific CTL should be comparatively analyzed in patients and healthy donors.

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