Brief Definitive Report

Human T Lymphocytes Recognize a Peptide of Single Point–mutated, Oncogenic ras Proteins

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Summary

P21ras proteins are thought to play an important role in cell proliferation and differentiation. Single nucleotide mutations in the encoding cellular proto-oncogenes often result in p21ras proteins with transforming activity. Such activated ras oncogenes have been demonstrated in a variety of human malignancies and also in preneoplastic changes. Using a synthetic peptide corresponding to amino acids 5–16 of mutated p21ras proteins with an exchange of the normal glycine at position 12 by valine, it is shown here that human CD4$^+$ T cells specifically recognize the mutated protein sequence and can be generated as antigen-specific T lymphocyte lines. The fact that these T lines did not crossreact to the sequence of normal p21ras proteins offers new perspectives for specific immunotherapy of human malignancies and even precancerous lesions.

The family of human ras proto-oncogenes (1) encodes membrane-bound proteins (p21ras), which are thought to be involved in signal transduction across the cell membrane. In this function, they most probably play a role in the regulation of cell growth and differentiation. Mutations in ras genes, which lead to proteins with increased activity, can be the molecular basis for the development of cancer. Single nucleotide mutations, which mainly occur in the codons 12, 13, and 61 of the ras proto-oncogenes, have been shown to result in malignant transformation of the cell (2, 3). Ras gene mutations have been identified in a variety of human cancers (4–7). The demonstration of tumor-specific protein sequences in human malignancies raises the question as to whether the immune system is able to recognize and to distinguish them from the normal protein.

Therefore, we analyzed the cellular immune response to a synthetic peptide (peptide 12val) representing amino acids 5–16 of mutated p21ras proteins with an exchange of the normal glycine at position 12 by valine. This peptide was chosen because in this region the products of the human Ha-ras, Ki-ras, and N-ras proto-oncogenes show identical amino acid sequences and because the substitution of glycine by valine induces transforming activity (2, 3) and is one of the most commonly found p21ras mutations in human tumors.

Materials and Methods

Antigens. Synthetic peptides representing amino acids 5–16 of normal p21ras proteins and the oncogene-encoded p21ras protein had the sequences KLVVVGAGGVGK (peptide 12gly) and KLV-VVGAVGVGK (peptide 12val), respectively. Peptides were prepared by Multiple Peptide Systems (San Diego, CA). PHA (Sigma Chemical Co., St. Louis, MO) was used in a concentration of 2.5 μg/ml.

Primary Responses and Generation of T Cell Lines. T lymphocyte lines specific for peptide 12val were established from peripheral blood samples of healthy individuals using a microculture approach. PBL were isolated from EDTA blood by centrifugation on Lymphoprep (Nygaard, Oslo, Norway) gradients. Washed PBL were resuspended in RPMI 1640 (Gibco Laboratories, Eggenstein, FRG) containing 1 mM glutamine (Gibco Laboratories), 1% MEM nonessential amino acids (100×) (Gibco Laboratories), 100 U/ml penicillin, 100 μg/ml streptomycin (Biochrom, Berlin, FRG), and 2% heat-inactivated FCS (Gibco Laboratories), referred to as complete medium. 2 × 10^5 cells in a volume of 100 μl/well were seeded into 96-well flat-bottomed microtiter plates (Nunc, Roskilde, Denmark). The peptide 12val was added to primary cultures in a final concentration of 10 μg/ml and cultures were incubated at 5% CO_2. Assays of the primary immune response were labeled after 4 d with 0.2 μCi [^{3}H]methyl-thymidine (Amersham, Braunschweig, FRG) per well and harvested 18 h later. At this time, cultures set up for the generation of T lines were provided with 100 μl/well of complete medium containing 15 U/ml human IL-2 (Lymphocult-HP; Biotest Diagnostics, Frankfurt, FRG) and 10% FCS. In intervals of 3–4 d, cultures were provided with fresh IL-2 containing medium without splitting.

Primary cultures were restimulated for the first time on day 16–18 by the split-well procedure. Cells were washed twice within their wells with 100 μl RPMI 1640. Cells of each well were resuspended in 100 μl complete medium, and twice, 50 μl of cell suspension from each well was transferred into two adjacent wells of a new microtiter plate. Only one of the two wells was provided with peptide 12val, but in both wells, 1.2 × 10^5 irradiated (4,000 rad) autologous PBL were seeded in a volume of 50 μl complete medium. 3 d later, cell growth in two adjacent wells derived from one single well was compared microscopically, and cultures with antigen-

specific cell proliferation were provided with IL-2-containing medium for further propagation. Exchange of IL-2 medium was again performed every third or fourth day and, when necessary, cultures were split.

In intervals of 14–16 d, cells were restimulated as described for proliferation assays. 72 h later, complete medium was replaced by IL-2-containing medium.

Proliferation Assays. 3×10^4 peptide 12val–generated T line cells were activated with 1.2×10^5 irradiated autologous PBL in the absence or presence of antigens or mitogen in a final volume of 120 μl. Cultures were labeled during the second day of restimulation with 0.2 μCi [^{3}H]methyl-thymidine (Amersham) per well and harvested 18 h later onto glass fiber filters by a cell harvester (Skatron, Inc., Sterling, VA). [^{3}H]Methyl-thymidine incorporation was measured by liquid scintillation counting. Data from triplicate cultures are given as arithmetic means.

Transfectants. Murine HLA-DR1-transfected fibroblasts (LDR1) as APC were kindly provided by J. Trowsdale, London. The transfectants were kept in RPMI containing 10% FCS and 1 mg Hygromycin B/ml (Sigma Chemical Co., St. Louis, MO). For proliferation assays, trypsinized and washed fibroblasts were incubated for 1 h at 37°C in RPMI containing mitomycin C (100 μg/ml) (Sigma Chemical Co.). Fibroblasts were washed three times and seeded at a density of 5×10^4 cells per well.

Antibodies. mAbs to monomorphic determinants of the human HLA-DP, -DQ, and -DR molecule were obtained from Becton Dickinson & Co. (Mountain View, CA). Before use, antibodies were dialyzed in PBS to remove sodium azide. 10 μl of antibody solution was added per well.

Results and Discussion

Existence of Peptide 12val–reactive T Lymphocytes in Normal Individuals. Immune responses to peptide 12val were analyzed in primary cultures of freshly isolated PBL derived from healthy persons. None of the four individuals investigated showed a significant primary response to the mutated protein sequence. In spite of this, T cell lines specific for peptide 12val could be established from the PBL of two healthy individuals. Maximal T line activation was induced by peptide 12val concentrations in a range from 0.3 to 30 μg peptide/ml (Fig. 1). These T cell lines, though derived from different donors, showed similar dose responses.

Peptide 12val–specific T Cells Are Not Autoreactive. Analysis of the T line responses to a second peptide (peptide 12gly), which represents the corresponding amino acids of unmutated p21ras proteins, revealed that none of the lines recognizing the sequence of the mutated protein (peptide 12val) crossreacted to the sequences of normal p21ras proteins (peptide 12gly) (Fig. 1).

Results of peptide competition experiments using suboptimal concentrations of peptide 12val (0.1 μg/ml) and peptide 12gly concentrations up to 100 μg/ml suggested that nonactivation of peptide 12val–specific lines is caused by nonpresentation of peptide 12gly by APC (not shown).

Peptide 12val–specific T Cells Are CD4$^+$ and MHC Class II Restricted. Antigen-induced proliferation of the peptide 12val line HZ.2, derived from a HLA-DR1 homozygous donor, could be blocked by antibodies directed to HLA-DR; the HLA-DR1 restriction of the line was further substantiated by presentation of peptide 12val by HLA-DR1-transfected fibroblasts (Fig. 2).

The phenotype of the peptide 12val–specific T lines was analyzed by cytofluorometry. All five lines investigated were composed of CD3$^+$ CD4$^+$ CD8$^-$ T cells bearing TCR-α/β (data not shown).

This study demonstrates that the immune system of normal individuals contains CD4$^+$ T lymphocytes specifically recognizing a protein sequence associated with malignant or preneoplastic cell transformation, and that it is possible to propagate these T lymphocytes in vitro using a peptide that corresponds to the highly conserved region of p21ras proteins with an oncogenic point mutation at position 12.

The fact that none of the T lines is activated by the sequence of the normal proto-oncogene product, thus are not autoreactive, offers new perspectives for specific immunotherapy of human neoplasms: either for adoptive immunotherapy with in vitro generated peptide 12val–specific autologous T cells or for active immunization with peptide 12val. Usage of peptides for the generation of T cell populations specific for oncogene-transformed cells may include the opportunity to generate cytotoxic MHC class II–restricted
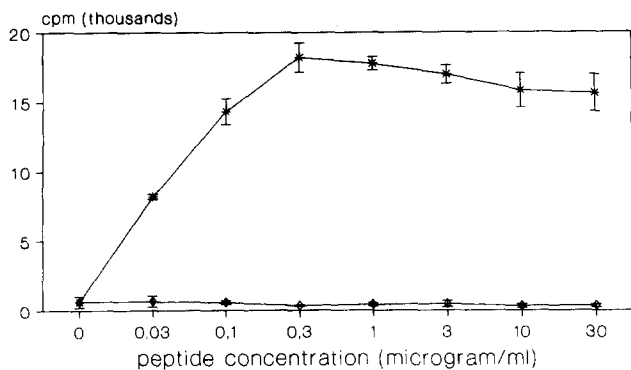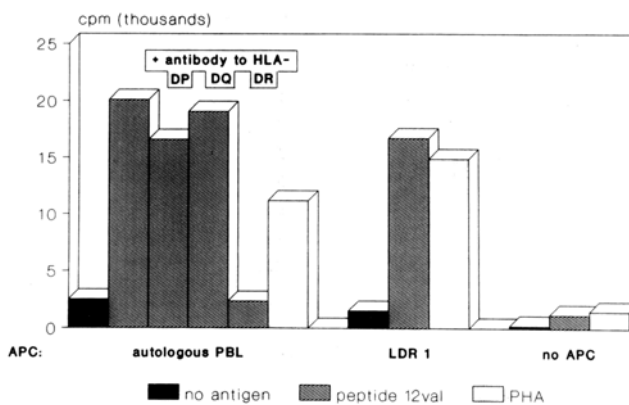

Figure 1. Activation of T cell line JA.12 by peptide 12val but not by peptide 12gly. [^{3}H]Thymidine incorporation of peptide 12val-specific T cells restimulated with irradiated autologous PBL in the absence or presence of indicated concentrations of peptide 12val (*) or peptide 12gly ($\Diamond$).

Figure 2. HLA-DR1 restriction of the peptide 12val-specific T cell line HZ.2. [^{3}H]Thymidine incorporation of peptide 12val-specific T cells restimulated with autologous PBL (*left set of columns*) or HLA-DR1-transfected murine fibroblasts (LDR1) (*second set of columns*).

CD4$^+$ T cells (8), as well as class I–restricted CD8$^+$ T lymphocytes (9–11); both cell types, at least in part (9), have been shown to be able to lyse also cells producing the recognized epitope endogenously (8, 10, 11). Also since proteins that are in their native state not expressed on the surface of the cell membrane can be recognized by T lymphocytes (12–14), oncogene-encoded protein sequences become attractive targets for immunotherapy (15).

In the murine and human system, the establishment of tumor-specific T cell lines has been described (16, 17), and it was shown that these populations, although of the CD4$^+$ phenotype, confer tumor-specific immunity (18, 19) and mediate eradication of established tumors (20, 21). The important role of CD4$^+$ lymphocytes in tumor immunity is further confirmed by in situ studies of the tumor environment (22).

T cells specific for ras oncogene–encoded proteins may have advantages in contrast to tumor-specific populations mentioned above, which are mainly reactive to tumor-specific transplantation antigens (TSTA) (17). A unique TSTA is only present on tumor cells derived from a single tumor, or a single cell, thus are individual for a certain tumor, in contrast to the sequence of mutated ras proteins, which occur in tumor cells of completely different origin. In addition, loss of TSTA-encoding genes by the tumor cells is a general feature and results, concerning this antigen, in nonimmunogenic tumor cells, which then can spread rapidly in the host (23). This cannot occur in oncogene-driven tumor cells since loss of the mutated oncogene would result in loss of tumorigenicity. In addition, T cells specific for the sequence of mutated p21ras protein would also be able to attack transformed cells in precancerous lesions, which have been shown to contain ras oncogene mutations as well (5, 24, 25).

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