Expression and Activity of Signaling Molecules in T Lymphocytes Obtained from Patients with Metastatic Melanoma before and after Interleukin 2 Therapy

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ABSTRACT

Recent studies have demonstrated altered expression and function of signaling molecules in T and natural killer cells in patients with cancer. The impairment of immune cell functions in advanced cancer may result from defects in signal transduction. We studied purified T cells obtained from peripheral blood or tumor-involved lymph nodes (LNs) of 45 patients with advanced metastatic melanoma for the presence of abnormalities in expression or activity of various signaling molecules. Western blot analyses demonstrated reduced expression of CD3- in 10 of 11 preparations of T cells obtained from tumor-involved LNs. Similar reduction in expression of CD3- was demonstrated by immunostaining performed in situ on frozen sections of melanoma tissues. Expression of p56 and Zap-70, but not phospholipase C-γ1, was reduced in these patients’ T cells relative to those obtained from normal individuals. In 50% of the patients, reduced expression of CD3- and p56 was observed in T lymphocytes obtained both from tumor-involved LNs and from peripheral blood. To determine whether deficient expression of these signaling molecules is reversible, T cells from melanoma-involved LNs were incubated in the presence of interleukin 2 (IL-2) for 48 h, and lysates from fresh or cultured lymphocytes were compared for changes in expression of signaling molecules. Cells cultured in the presence of IL-2 demonstrated increased expression of CD3- and p56, which approached the levels detected in normal T cells. However, the level of p56 kinase activity did not normalize in any of the LN-derived lymphocytes cultured in the presence of IL-2. Decreased expression of CD3- or p56 observed in the patients’ T cells was not reversed by immunotherapy with IL-2 at low or high dose in those patients with metastatic melanoma who failed to respond to therapy. However, in three patients who achieved clinical responses, the initially reduced expression of CD3- in peripheral blood T cells normalized following IL-2 therapy.

INTRODUCTION

Lymphocytes infiltrating various human tumors, including melanoma, express activation markers (1) and display a restricted usage of TcR V-gene products (2–4). Recently, the genes for several human melanoma antigens have been cloned, and the corresponding tumor-specific antigens have been shown to be recognized by class I MHC-restricted T lymphocytes (5). However, T lymphocytes infiltrating human solid tumors appear to be unable to control tumor growth. Combined experience from many laboratories clearly indicates that T cells freshly derived from the tumor microenvironment are immunosuppressed. TILs show variable but significant decreases in proliferative responses at the population as well as single-cell levels (6, 7), in cytotoxicity (8), migration (9), and the ability to produce certain cytokines (10). However, when cultured in the presence of IL-2, these TILs become capable of mediating antitumor activity, as assessed in cytotoxicity assays or by production of cytokines in response to autologous tumor (11, 12).

Several possible mechanisms have been suggested to account for the lack of immune competence of freshly isolated TILs, including clonal deletion (13), development of suppressor lymphocytes or macrophages (14), tolerance induced by tumor cells that fail to deliver costimulatory signals (15), aberrant antigen presentation (16), down-regulation of expression of MHC molecules required for peptide presentation (17), or production of suppressor factors by tumor cells (7). Studies from our and other laboratories indicated that alterations in expression and functions of signal transduction molecules associated with the TcR may be responsible for deficient immune responsiveness of T cells in several types of malignancy. Fresh TILs and, in some cases PBLs, obtained from renal cell carcinoma (18, 19), colorectal carcinoma (20, 21), ovarian carcinomas (22), or melanoma (23) showed marked decrease in CD3-chain expression. This reduced expression of the CD3 chain was detectable by various methods, including Western blotting (18, 21–23), CD3-specific ELISA (22), flow cytometry analysis of permeabilized T cells (20, 21, 24), or immunostaining of tumor sections (18). In ovarian carcinoma and melanoma, the altered

2 The abbreviations used are: TcR, T-cell receptor; TIL, tumor-infiltrating lymphocyte; PBL, peripheral blood lymphocyte; IL, interleukin; LN, lymph node; LNL, lymph node lymphocyte; UPCI, University of Pittsburgh Cancer Institute; mAb, monoclonal antibody; PLC, phospholipase C; GAM1g, goat anti-mouse immunoglobulin; PTK, protein tyrosine kinase.

signaling capability of T cells was associated with reduced cellular functions and decreased expression of genes encoding the Th1 cytokines, IFN-γ and IL-2 (22, 23). In TILs of patients with renal cell carcinoma, the level of ζ chain expression was found to positively correlate with the ability of these cells to produce TNF-α (19). In colorectal carcinoma, the levels of ζ expression in T cells was shown to directly correlate with the distance from the tumor and with the stage of the disease (21). Correlation between reduced expression of ζ and the disease stage was also observed in a study performed with PBLs obtained from 55 patients with various types of carcinoma (25). In melanoma patients, the overall survival was significantly shorter in patients with a low level of expression of ζ chain in peripheral blood T lymphocytes compared to that of patients with a normal level of ζ expression (23).

Evidence for signaling defects in experimental models of tumor metastasis has been conflicting (26-28). However, in mice bearing fibrosarcomas, reduced levels of TCR-ζ, p56Lck and p59Fyn expression, as well as calcium mobilization in response to CD3 cross-linking were observed in spleen T cells. In mice bearing IL-2-transduced fibrosarcomas secreting IL-2, these alterations in signal transduction were not present (29). Also, functional defects were found only in T cells of mice bearing the parental but not IL-2-secreting tumors (30). Thus, the presence of IL-2 produced at the tumor site prevented or reversed alterations in expression of signaling proteins as well as hypersensitivity.

In view of these results, we examined effects of exogenous IL-2 on expression and activity of TCR-associated signaling molecules in human T cells obtained from patients with advanced melanoma. Our initial studies demonstrated marked alterations in expression and function of signaling proteins in most preparations of T lymphocytes isolated from tumor-involved LNs of these patients. In contrast, only 50% of the cases had signaling defects in T lymphocytes obtained from matched peripheral bloods. Short-term in vitro exposure of LNLs to IL-2 did not always result in the recovery of signaling capability. However, clinical responses to IL-2 therapy observed in three patients appeared to be associated with reversal of the reduced expression of CD3-ζ documented prior to therapy.

MATERIALS AND METHODS

Patients. All patients studied had metastatic melanoma and were participants of or were evaluated for entry into Phase 1b clinical trials with IL-2 at the UPCI between 1987 and 1994. At the time of this study, all protocols were completed, and cryopreserved cells were utilized for experiments reported below. Cryopreservation was not found to adversely affect the level of expression of intracellular proteins. Furthermore, normal PBLs used as controls in signal transduction experiments were cryopreserved as well. All 30 patients with melanoma who contributed LNLs to this study were evaluated and found to be ineligible for adjuvant IFNα-2b therapy, as a part of Institutional Review Board-approved UPCI protocol 91-70 (J. M. Kirkwood, principal investigator). Their LNLs and PBLs were submitted to and banked by the UPCI Tissue Procurement Facility. These patients had lymph node metastases, for which staging or therapeutic dissection was undertaken, but were found to be ineligible for entry into the adjuvant trial due to the presence of more extensive regional/distant disease or other temporal factors.

The patients who were treated with low-dose IL-2 (n = 7) were participants in the in vitro sensitization protocols (T. F. Logan, principal investigator). The patients were immunized with irradiated autologous tumor cells and Bacillus Calmette-Guérin, had LNs draining the vaccination site removed, and received a single adoptive transfer of in vitro-sensitized LNLs plus IL-2 (Hoffmann-La Roche, Inc.) given as bolus injections every 8 h × 15 at 30,000 units/kg of body weight (1 × 10⁶ units/m²). The patient (M. W.), who was a complete responder to IL-2, did not receive a transfer of LNLs and was treated with IL-2 only, as described above.

The patients treated with high-dose IL-2 participated in a Phase 1b protocol that included adoptive transfer of IL-2-activated natural killer cells plus IL-2 (Cetus) administered as continuous infusions at the dose of 1 × 10⁶ units/m²/day × 5. An additional 5-day course of IL-2 alone was started on day 18. The patients who had objective clinical responses were eligible for additional courses of adoptive immunotherapy and IL-2 (31). Of 15 patients eligible for this protocol, 11 had melanoma. Cells from eight of these patients were available for this study.

Isolation of Lymphocytes from Tumor-involved LNs. Lymphocytes were isolated from fresh specimens of surgically removed tumor-involved LNs from patients with metastatic melanoma (n = 30) as described earlier (2). Briefly, tumor tissues were minced into 1-mm³ pieces, which were then dissociated in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated FCS, 0.05% collagenase (type 4; Sigma Chemical Co., St. Louis, MO), and 0.002% DNase (type 1; Sigma) on a magnetic stirrer for 1 h. Subsequently, the tissue digests were washed and passed through a nylon mesh, and LNLs and tumor cells were separated on discontinuous (75%/100%) Ficoll-Hypaque gradients. The percentage of CD3⁺ T cells obtained from tumor-involved LNs with no further purification ranged from 65 to 94% (73 ± 3, mean ± SEM).

Purification of T Cells from Peripheral Blood Mononuclear Cells or LNLs. Mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation from leukapheresis products obtained from platelet donors (Central Blood Bank of Pittsburgh, Pittsburgh, PA) or from heparinized blood obtained from patients at the same time as the involved LN. To select for PBLs, monocytes were removed on nylon wool columns as described previously (32). T cells were purified from PBLs or LNLs by negative selection as described earlier (22). Briefly, PBLs were incubated in the presence of anti-CD16, anti-CD19, and anti-CD14 mAbs (10 µg/ml; Dako Co., Carpinteria, CA) for 30 min at 0°C. The cells were then washed twice and incubated with magnetic beads coated with goat antimouse immunoglobulins (1 cell:30 beads; PerSeptive Diagnostics, Cambridge, MA) for 30 min at 0°C. After each of two successive incubations with magnetic beads, a magnet was used to separate beads with attached cells. Two-color flow cytometry was used to determine the purity of the negatively selected T-cell populations. Purified T cells obtained by negative selection from either PBLs or LNs contained more than 90% CD3⁺ cells. Cryopreserved PBLs or PBL-T cells from normal donors were used as controls for cryopreserved specimens from patients.
A. 

Fig. 1 Expression of CD3-ζ and CD3-ε (A) and Lck (B) in T lymphocytes obtained from the peripheral blood or tumor-involved LNs of patients with metastatic melanoma as compared to peripheral blood T lymphocytes from normal (N) donors. Lysates of 2.5 × 10⁶ T lymphocytes/lane were electrophoresed in 12% SDS gels. The separated proteins were electrophoretically transferred to a nitrocellulose membrane and blotted with anti-CD3-ζ or anti-CD3-ε (both at 1:1000 dilution). Experiments performed with cells obtained from 7 of 11 patients studied for ζ and 4 of 9 studied for Lck are shown.

B. 

Western Blotting. Cell preparations from normal donors and patients were lysed by incubation in lysis buffer supplemented with a high concentration of protease inhibitors (50 mM HEPES (pH 7.2), 150 mM NaCl, 5 mM EDTA, 1 mM orthovanadate, 2.5% Triton X-100, 200 μg/ml trypsin/chymotrypsin inhibitor (Sigma, St. Louis, MO), 200 μg/ml chymostatin (Boehringer Mannheim, Indianapolis, IN), and 2 mM PMSF (Sigma)] for 20 min at 4°C. This combination of protease inhibitors has been demonstrated to completely inhibit granulocyte proteases that might induce nonselective protein degradation in vitro (26). The lysate was centrifuged at 14,000 rpm for 10 min, and postnuclear supernatant was harvested and sampled for quantitation of protein concentration, utilizing Bio-Rad DC protein assay (Hercules, CA). The rest of the lysate was then mixed with an equal volume of 2× SDS-PAGE sample buffer, boiled for 5 min, and either stored at −70°C for later use or immediately subjected to electrophoresis. Cellular lysates obtained from a similar number of patients versus normal T lymphocytes were electrophoresed in 12% (for CD3-ζ or CD3-ε), 10% (for Lck), or 7.5% (for PLC-γ1) SDS gels under reducing conditions (22). The separated proteins were then electrophoretically transferred to polyvinylidene difluoride membrane Immobilon-P (Millipore, Bedford, MA). Following blocking by 3% solution of nonfat dried milk and 1% BSA for 1 h at room temperature, the membranes were blotted with anti-ζ antisera generously provided by Dr. R. Robb (OncoTherapeutics, Cranberry, NJ), anti-CD3-ε (Dako), and anti-p56κκ or anti-Zap-70 (UBI, Lake Placid, NY) antisera, all used at a 1:1000 dilution. Optimal dilutions of the antibodies were determined in titration experiments, using serial antibody dilutions to blot the same cellular lysate. After washing in Tris-buffered saline with 0.05% Tween 20, the membrane was incubated with antirabbit immunoglobulin-horseradish peroxidase (1:5000 dilution; Amersham, Arlington, IL) for 1 h. Protein bands were detected by enhanced chemiluminescence (ECL; Amersham). Intensity of expression was determined by densitometry reading (Molecular Dynamics).

In Vitro Protein Tyrosine Kinase Assay. Freshly purified T cells from normal donors or melanoma patients (5 × 10⁶/sample) were treated with anti-CD3 mAb (UCHT1; Immunochem, Westbrook, ME) for 30 min on ice. Cells were washed twice with cold RPMI supplemented with 2% FCS and incubated with 20 μg/ml F(ab)2 fragments of GAMlg as secondary cross-linking antibody for 5 min at 37°C. Stimulated cells were lysed at 4°C for 30 min, and postnuclear supernatant was harvested after centrifugation at 14,000 rpm at 4°C for 10 min.

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Richard Robb, personal communication.
Lysates used for these studies were prepared from equal numbers of purified cells and confirmed to contain equivalent protein concentrations. Anti-p56κκ mAbs, at a concentration of 2 μg/100 μl/5 × 10⁶ cells, were used to immunoprecipitate the tyrosine kinase from cell lysates (33). The immunoprecipitates were washed three times with each of the following buffers: PBS/1% NP40, Tris-LiCl, and Tris-NaCl-EDTA. Kinase buffer [50 mM Tris (pH 7.4), 10 mM MnCl₂, and 10 mM MgCl₂], 10 μM ATP, and 10 μCi/sample of [γ-³²P]ATP (DuPont NEN, Boston, MA) were added and incubated for 20 min at room temperature. The reaction was stopped by the addition of 2× Laemmli buffer. The samples were then boiled and loaded onto a 10% SDS-polyacrylamide gel, followed by autoradiography.

**Immunohistology.** An immunoperoxidase procedure was performed as described previously (34) on frozen sections of melanoma or control normal skin, stained with mAb to CD3 (25 pg/ml, TIA-2, IgGl; Coulter, Hialeah, FL) or antiserum specific for CD3-ε (10 pg/ml; Dako). As positive controls, purified normal human T cells and T cells infiltrating the renal tissue obtained from a patient who rejected the kidney transplant were stained with ζ- or ε-specific Abs. Negative controls included cells or tissue sections treated with PBS and normal rabbit serum or mouse IgG as isotype control.

**Flow Cytometry.** Analysis of cell surface phenotype was performed by direct staining with FITC- or phycoerythrin-conjugated mAbs purchased from Becton Dickinson (Mountain View, CA). Assessment of ζ expression by flow cytometry was performed on lymphocytes fixed in 0.5% paraformaldehyde in HBSS for 20 min on ice and then permeabilized by digitonin (10 μg/ml) for 5–10 min on ice (35). The degree of permeabilization was assessed by trypan blue uptake. The cells were resuspended in HBSS containing 0.05% Tween 20 and blocked with mouse IgG (20 μg/ml) for 20 min on ice. After two washes of excess IgG, the cells were stained with mouse anti-ζ mAb (TIA-2) or IgG1 isotype control at the same concentration for 30 min on ice. The cells were washed three times with HBSS containing 0.05% Tween 20 and then incubated with FITC-conjugated GAMg for 20 min on ice.

**RESULTS**

**Expression of Signaling Proteins in T Lymphocytes Isolated from Tumor-involved LNs.** To investigate expression of signal transduction proteins in T cells recovered from the tumor microenvironment or distant from the tumor, we have used LNs involved with melanoma cells (50–80% involvement) or peripheral blood T cells from the same melanoma patients, respectively. As a control, peripheral blood T lymphocytes from normal donors were used. In performing Western blotting, it was important to ensure that the same quantity of cellular proteins, solubilized from equivalent numbers of patients versus normal T cells, was applied to the gels. For this reason, purified T cells with >90% CD3⁺ phenotype were used in most experiments. The lysates prepared from equivalent numbers of these purified T cells were quantitated for the protein content, and the same amount of protein was used for each lane of the gel. In samples containing fewer than 90% CD3⁺ cells, as assessed by flow cytometry, lysates applied to various lanes were prepared from an equivalent number of patients versus normal T cells. Western blot analyses were performed to examine the level of expression of signaling molecules known to be activated via

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**Fig. 2** Expression of Zap-70 (A) and PLC-γ1 (B) in T lymphocytes obtained from the peripheral blood or tumor-involved LNs of patients with metastatic melanoma as compared to peripheral blood T lymphocytes from normal (N) donors. Lysates of 2.5 × 10⁶ T lymphocytes/lane were electrophoresed in 10 or 7.5% SDS gels. The separated proteins were electrophoretically transferred to a nitrocellulose membrane and blotted with anti-Zap-70 or anti-PLC-γ1 (both at 1:1000 dilution).
Fig. 3  Immunoperoxidase staining of serial cryosections of normal human skin (A, B, and C) and melanoma (D, E, and F) with anti-CD3-ζ mouse mAb (A and D) or rabbit anti-CD3-ε antibody (B and E) and normal mouse and rabbit immunoglobulin controls (C and F). Note intensity of positive T cells in the epidermis and in connective tissue of the dermis in both A and B. Few cells are stained for ζ chain in D, compared to intense staining of T cells positive for the ε chain in E. ×1000.

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Reduced expression of Lck was also observed in all nine tested preparations of LNLs obtained from patients with metastatic melanoma. In contrast to CD3-ζ protein, which in several cases appeared to be absent by Western blotting, expression of Lck was reduced but always detectable by this method (Fig. 1B). In PBL-T cells, expression of Lck was reduced in four of eight melanoma patients studied. LNLs obtained from melanoma patients also demonstrated a reduced expression of Zap-70, which was not detectable by Western blot analysis, under identical experimental conditions used to detect a strong signal in normal PBLs (Fig. 2A). However, expression of PLC-γ1 in LNLs obtained from melanoma patients was not altered as compared to that in normal PBLs. These observations suggest that in patients with metastatic melanoma, selective reduction in expression of only certain proteins associated with signal transduction via the TcR occurs in the tumor microenvironment.

In Situ Immunostaining of Melanoma Tissues. To exclude the possibility that the observed reduced expression of CD3-ζ in lymphocytes isolated from tumor-involved tissues was attributable to degradation induced during in vitro processing, we performed immunostaining of fresh melanoma tissues. Cryo-
sections of melanoma as well as control normal tissues were strongly stained with anti-CD3-ε antibody (Fig. 3, B and D). In contrast, expression of CD3-ζ as assessed by the number of positive cells or level of staining was markedly decreased in melanoma tissues, as compared to that in normal tissues used as controls (Fig. 3, A and C). Furthermore, T lymphocytes in melanoma tissues, which were strongly stained with anti-CD3-ε, were either negative or only weakly stained by CD3-ζ-specific mAb in serial tissue sections. Results of immunostaining for CD3-ζ indicate that the reduced expression of the CD3-ζ observed in Western blots is also detectable in situ and thus cannot be attributed to in vitro proteolytic degradation during the preparation of cell lysates.

Modulation of Expression of Signaling Molecules in T Cells by Culture with IL-2. To test the possibility that the observed defects in expression of the signaling molecules might be reversible, T cells isolated from tumor-involved LNs were cultured in the presence of IL-2 (60-1500 IU/ml) for 48 h. Lysates from fresh or cultured T lymphocytes were compared for changes induced during culture in expression of signaling elements. Cells cultured with IL-2 had increased expression of CD3-ζ in two of five and of Lck in four of five patients (Fig. 4, A and B). In two of five patients studied, expression of Lck was increased also in cells cultured in medium alone (Fig. 5, A and B). Thus, in some cases, removal of tumor cells led to reversal in expression of Lck. The culture conditions utilized did not affect the levels of expression of either CD3-ζ or Lck in T cells from peripheral blood of normal donors. Similarly, expression of PLC-γ1, which was not reduced in LNLs from melanoma patients, was not affected by culture in the presence of IL-2 (Fig. 4C).

Lck Activity in the Patients’ T Lymphocytes. In T lymphocytes, the activity of Lck is usually triggered by stimulation via TcR, which induces the physical and functional association of Lck with the CD4 or CD8 co-receptors (36). We, therefore, evaluated the functionality of the TcR on T cells purified from melanoma-involved LNs by measuring its ability to activate the PTK Lck following receptor cross-linking by CD3-specific mAb. In comparison to T cells purified from peripheral blood of normal donors, reduced or undetectable kinase activity was observed in all seven tested preparations of purified T cells isolated from melanoma-involved LNs (Fig. 6A). Lck activity measured as the ratio between [32P]Lck in CD3-cross-linked cells and control cells, which were not cross-linked, was significantly higher \( P < 0.01 \) in T cells from normal peripheral blood \( 2.9 \pm 0.4; n = 9 \) than in T cells isolated from melanoma LNs \( 0.8 \pm 0.2; n = 7 \). The reduced activity of Lck in melanoma LNLs probably correlated with its reduced expression, as indicated by Western blotting (Fig. 1). Incubation in the presence of IL-2, and in several cases in

Fig. 4 Effects of in vitro culture in the presence of IL-2 (1500 IU/ml) for 48 h on expression of CD3-ζ (A), Lck (B), or PLC-γ1 (C) in T cells purified from tumor-involved LNs of melanoma patients as compared to PBLs or PBL-T cells obtained from normal (N) donors. Expression was analyzed by Western blotting as described in the legend to Fig. 1.

Fig. 5 Effects of in vitro culture in medium alone or in the presence of various concentrations of IL-2 for 48 h on expression of Lck (A and B) and on activity of Lck (C) in T lymphocytes purified from tumor-involved LNs of melanoma patients as compared to PBL-T cells from normal (N) donors. Expression of Lck was assessed by Western blotting as described in the legend to Fig. 1, and activity of Lck was determined in immune complexes as described in the legend to Fig. 6.
medium alone, enhanced the level of expression of Lck (Figs. 4 and 5). Therefore, it was important to determine whether this recovery in expression of Lck was associated with normal levels of kinase activity. As shown in Figs. 5, B and C, and 6A, incubation of LN-derived T lymphocytes in the presence of IL-2 for 48 h did not lead to recovery of normal levels of Lck activity upon cross-linking of TcR with anti-CD3 mAbs. Furthermore, reversal in the level of expression of Lck was observed in four of five specimens tested, whereas a normal level of activity was not detected in any of seven other samples tested following incubation with IL-2. Therefore, it appears that the functional potential of TcRs to induce Lck activity was not fully regained after culture in IL-2, despite the recovery in Lck expression. In contrast to T cells purified from melanoma-involved LNs, which were all deficient in Lck activity, those purified from the same patients’ peripheral blood were not always affected (Fig. 7). Deficient activation of Lck was observed in only three of seven T-lymphocyte preparations obtained from PBLs of melanoma patients, following triggering by CD3-specific mAb.

Expression of Signaling Molecules in T Lymphocytes of Patients Treated with IL-2. To study the in vivo effects of IL-2 therapy on expression of signaling molecules in circulating T lymphocytes in patients with metastatic melanoma, we have used cryopreserved PBLs from patients on two different IL-2 therapy protocols. Seven patients were treated with adoptively transferred, in vitro-sensitized LNLs plus a course of low-dose IL-2 as described in “Materials and Methods.” Their PBLs were tested for expression of CD3-ζ and p56^Lck (Fig. 8). In comparison to PBLs of normal donors, expression of CD3-ζ in PBLs obtained from three of seven patients studied was reduced at the start of the therapy (PBL-1) and was not increased when evaluated 1 day (PBL-2) or 1 month after administration of IL-2 (PBL-3). Of the three patients who demonstrated reduced expression of CD3-ζ, only two also demonstrated reduced expression of p56^Lck that, similar to expression of CD3-ζ, was not corrected by the therapy (Fig. 8B). However, in a single melanoma patient (M. W.), who responded with a complete remission to low-dose IL-2 therapy, expression of CD3-ζ was reduced prior to therapy and on day 41 after one course of IL-2 administration but appeared to be normal when tested in cells harvested 6 years later (Fig. 9). This patient remains in remission 6 years after therapy.

We have also tested in vivo effects of high-dose IL-2

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**Fig. 6** In vitro PTK assays for p56^Lck in T cells freshly obtained from tumor-involved LNs of patients with melanoma (A) or cultured in the presence of IL-2 (1500 IU/ml) for 48 h (B). T lymphocytes purified by negative selection from tumor-involved LNs of melanoma patients were compared to PBL-T cells from normal (N) donors. T cells at 5 × 10^6/sample were treated with anti-CD3 (10 μg/ml) for 30 min on ice. Cells were washed twice with cold medium and incubated with 20 μg/ml F(ab')_2 fragments of GAM Ig as secondary cross-linking antibody at 37°C for 5 min. Following lysis, Lck was immunoprecipitated by rabbit antihuman Lck (NH2-terminal) antibody and protein A-Sepharose beads. The kinase reaction was carried out in the presence of 1 μM ATP and 10 μCi [γ-32P]ATP. Reaction products were resolved by electrophoresis, and dried gels were subjected to autoradiography and 32P quantitation by PhosphorImager. Experiments performed with cells obtained from three of seven patients studied are shown.
therapy on expression of CD3-ζ in circulating T lymphocytes obtained from eight patients with melanoma. Marked reduction in expression of CD3-ζ, as assessed by Western blotting, was observed at the start of IL-2 therapy in six of eight patients tested (G. L., C. H., M. O.; C. R., M. F., K. K.; Fig. 10). Following IL-2 administration, increased expression of CD3-ζ was observed in the two patients (G. L. and C. H.), who also responded clinically. No change in expression of CD3-ζ following IL-2 therapy was observed in the five other patients (R. D., B. B., M. O., C. R., and M. F.) who did not respond clinically. In one patient (K. K.), an increased expression of ζ observed on day 35 after IL-2 administration was not associated with clinical response. These results were confirmed by testing expression of CD3-ζ by flow cytometry of T cells permeabilized with digitonin. The results obtained with permeabilized T cells obtained from a partial responder (C. H.) to IL-2 therapy and a nonresponder are shown in Fig. 11. A marked reduction in the level of CD3-ζ expression in T cells obtained before therapy was observed in both patients. As can be seen in Fig. 11A, expression of CD3-ζ in patient C. H. almost normalized following IL-2 therapy. In contrast, the reduced expression of CD3-ζ seen in peripheral blood T lymphocytes of patient M. O., a nonresponder, remained decreased following therapy.

**DISCUSSION**

The presence of signaling defects in the TcR-CD3 pathway in T lymphocytes obtained from patients with advanced cancer has been now reported by several laboratories (18–24). Initially observed in splenocytes of long-term tumor-bearing mice (28), these defects appear to be consistently present in tumor-associated T lymphocytes and natural killer cells recovered from patients with metastatic disease (18, 20, 22, 25, 26). The presence of signaling defects provides a partial explanation for previous observations accumulated over the years that functional abnormalities exist in T cells of patients with cancer, especially in T cells isolated from the tumor microenvironment (1, 6–10). These defects are detectable as decreased expression and function of various signaling molecules but are not all-or-none phenomena. The defects are often variable and may not occur at all in some patients. The same observations have been made in respect to proliferation or cytolytic functions of fresh-ly-isolated TILs in patients with melanoma, renal cell carcinoma, and other tumors (1, 6–10).

The mechanisms responsible for the observed defects in signaling are unknown. In view of consistent reports that immune defects in lymphocyte functions are particularly pronounced in the tumor microenvironment and may not be detectable in peripheral blood T lymphocytes (1, 6–10), it has been assumed that human tumors are a source of immunosuppression. Considerable evidence exists, documenting the ability of human tumors to produce a variety of soluble factors capable of interfering with proliferative and cytolytic functions of human lymphocytes (7, 37–39). It is possible that the same factors may also contribute to tumor-induced T-cell anergy by inducing or sustaining signaling defects.
A.

Fig. 8 Expression of CD3-ζ (A) and Lck (B) in T lymphocytes obtained from melanoma patients before and after therapy with adoptively transferred, in vitro-sensitized LNLs and a course of low-dose IL-2. Tumor-involved LNs and PBL-1 were obtained before the therapy, whereas PBL-2 and PBL-3 were obtained 1 day after the course of IL-2 administration or 1 month after IL-2, respectively. PBLs from normal (N) donors served as positive controls. Expression of Lck was evaluated by Western blotting as described in the legend to Fig. 1.

B.

In vitro activation of human TILs and their proliferation in the presence of IL-2 has been reported previously (40). However, TILs obtained from some human tumors failed to proliferate, even in the presence of high doses of IL-2 (41). We also observed that TILs isolated from metastatic sites proliferated less well or not at all in the presence or IL-2, relative to TILs obtained from primary tumors (42). It was, therefore, of interest to determine the effects of IL-2 on signaling defects in T lymphocytes of patients with metastatic melanoma in vitro and in vivo. Some of the T lymphocytes obtained from melanoma-involved lymph nodes demonstrated normalization in expression of the ζ chain or Lck following incubation in the presence of IL-2 for 48 h. The possibility that this normalization was a consequence of outgrowth of T cells stimulated in the presence of IL-2 cannot be excluded. Although in several lymphocyte cultures IL-2 appeared to mediate the enhanced level of ζ chain or Lck expression, in other cases removal of tumor cells and culture in FCS-supplemented medium alone induced reversal in Lck expression. As this reversal was rapid and occurred in the absence of IL-2 in some cultures, it does not seem to be a result of cell proliferation. It is more likely that deficient T cells regain expression of ζ or Lck under favorable in vitro conditions. However, the observed normalization of expression of signaling molecules after culture in the presence of IL-2 or in medium alone did not translate into the recovery of activity of Lck inducible via TcR. These results suggest that decreased PTK activity is not only a consequence of decreased expression of these proteins but represents a functional impairment of TcR-mediated signal transduction.

Low-dose therapy with IL-2 performed as a part of the Phase Ib “in vitro sensitization” protocol did not appear to reverse decreased expression of signaling molecules in patients with metastatic melanoma. Furthermore, normalization of the ζ chain expression was not observed in most patients with metastatic melanoma who were treated with high-dose IL-2 therapy. However, melanoma patients who had decreased expression of the ζ chain in peripheral blood T cells prior to treatment and who were responders to low- or high-dose IL-2 therapy demonstrated normalization of ζ. This observation suggests that normalization in expression of ζ chain in patients with melanoma is a favorable sign. Notably, the patient who achieved remission after low-dose IL-2 therapy and remains disease free shows normal ζ chain expression 6 years after therapy. Normalization of ζ chain expression after immunotherapy in these responders observed in Western blots was confirmed in two cases by flow cytometry, using permeabilized peripheral blood T cells. Flow cytometry appears to be more sensitive than Western blots for detection of abnormalities in expression of the ζ chain, and it can measure partial reversals of ζ chain expres-
Signaling Deficiency in T Cells from Patients with Melanoma

Days post IL-2: 0 35 84 0 35

Days post IL-2: 0 35 0 35 0 35

Days post IL-2: 0 35 0 29 0 35

Fig. 10. Expression of CD3-ζ in PBLs of melanoma patients treated with high-dose IL-2. PBLs were obtained before and after therapy and compared to PBLs from normal (N) donors. Clinically, one patient demonstrated complete response (CR), one demonstrated partial response (PR), and six were nonresponders (NR). Expression was analyzed by Western blotting as described in the legend to Fig. 1. Densitometry readings of expression in patients’ PBLs relative to normal controls (% of normal expression before and after therapy) are as follows: GL, 10 and 50; CH, 0 and 30; RD, 50 and 40; BB, 60 and 50; MO, 10 and 0; CR, 0 and 5; MF, 2 and 5; and KK, 10 and 40.

Fig. 11. Flow cytometric analysis of expression of CD3-ζ in PBLs from patients with metastatic melanoma before and after administration of high-dose IL-2. PBLs from normal donors served as positive control. The cells were fixed, permeabilized, blocked with nonspecific mouse IgG, and stained with anti-ζ mAb, followed by FITC-conjugated GAMlg. Control cells were stained with IgG1 isotype control and FITC-GAMlg.

These new observations are potentially very important, because they suggest that it may be possible to predict clinical responses to IL-2 therapy in patients whose T cells show signaling defects. In spite of extensive immunological monitoring performed in patients with melanoma or other cancers who were treated with IL-2 (1, 40, 43), no single in vitro correlate of response to biotherapy has been established. Several years ago, Aebersold et al. (43) reported that in patients with melanoma receiving TIL and IL-2 therapy, the ability of circulating lymphocytes to lyse autologous melanoma significantly correlated with clinical responses to this therapy. However, substantial practical difficulties exist with monitoring biotherapy by the use of autologous tumor cells for cytotoxicity assays. In contrast, the presence of signaling defects in peripheral blood lymphocytes and normalization of these defects in response to therapy can be easily monitored by flow cytometry or several other techniques routinely available in most laboratories.

Overall, our data indicate that a range of signal transduction defects occurs in patients with metastatic melanoma and demonstrate normalization of expression of one or perhaps more signaling molecules in T cells of patients who have had a favorable response to therapy with IL-2. These data are intriguing and indicate a need for a more comprehensive evaluation of signaling defects in lymphocytes of a large cohort of cancer patients treated with biological response modifiers. A study focused on alterations in T-cell signal transduction before, during, and after therapy may provide better insights into the clinical significance of these changes. It is possible that normalization of ζ-chain expression may prove to be an in vitro predictor or surrogate of response to IL-2 and thus of use in monitoring of immunological therapies including this cytokine.

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