Interleukin-12 Augments Cytolytic Activity of Peripheral Blood Lymphocytes From Patients With Hematologic and Solid Malignancies

By Robert J. Soiffer, Michael J. Robertson, Christine Murray, Keith Cochran, and Jerome Ritz

Interleukin-12 (IL-12) is a heterodimeric 70-kD cytokine that can enhance the activity of cytotoxic effector cells. Although IL-12 shares some functional properties with interleukin-2 (IL-2), it appears to act via a distinct mechanism. In this report, we examined the effects of IL-12 on the cytolytic activity and proliferation of peripheral blood mononuclear cells (PBMC) obtained from patients with malignant disease. PBMC from two groups of patients were evaluated. The first group consisted of 12 individuals with metastatic solid tumors. PBMC from these patients demonstrated a marked defect in their ability to lyse natural killer (NK)-sensitive targets (K562) compared with normal volunteers. Overnight incubation with IL-12 (35 pmol/L) corrected this defect. The effect of 35 pmol/L of IL-12 on cytotoxicity was similar to that of 3 nmol/L of IL-2. In contrast, this concentration of IL-12 had little effect on cytolytic activity against an NK-resistant cell line (COLO 205). When IL-12 was added to PBMC obtained from cancer patients who were being treated with low-dose IL-2 in vivo, a dramatic increase in cytolytic activity against both NK-sensitive and -resistant tumor targets was observed. Unlike IL-2, IL-12 failed to stimulate proliferation of resting PBMC from cancer patients significantly. The second group of patients we studied comprised 13 patients who had recently undergone allogeneic bone marrow transplantation (BMT) for hematologic malignancy. In resting PBMC from these transplant recipients, IL-12 was capable of enhancing cytotoxicity against both NK-sensitive and -resistant tumor targets. Our findings indicate that IL-12 can restore defective NK activity of PBMC from patients with metastatic cancer, as well as enhance cytolytic function of PBMC from patients after allogeneic BMT. The clinical use of IL-12 as an immunomodulator in patients with malignancy merits further consideration.

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MATERIALS AND METHODS

Patient samples. Blood samples were obtained from patients at the Dana-Farber Cancer Institute (DFCI). All patients were entered onto clinical trials approved by the Human Subjects Protection Committee at DFCI. Samples were derived from several different groups of patients and were analyzed separately. The first group included 12 patients with metastatic cancer who had not received chemotherapy or radiation therapy for at least 8 weeks before sample collection. Additional samples were obtained at a later date on two of six of these patients during the administration of low-dose IL-2 (4.5 × 10^5 U/m^2/d) via continuous infusion as part of a DFCI clinical protocol. These samples were taken after patients had received IL-2 daily for 4 to 6 consecutive weeks. Control samples were obtained from healthy volunteers.

Samples were also obtained from patients who had undergone allogeneic BMT at DFCI for hematologic malignancies. All patients received marrow depleted of mature T lymphocytes with monoclonal antibody (T12) recognizing the CD6 surface antigen plus complement.29 No patients were receiving any additional immune suppressive agents for graft-versus-host disease (GVHD) prophylaxis, and none had active GVHD at the time of sample collection. Blood was obtained on these patients 3 to 5 weeks after marrow infusion. Immunophenotypic analysis. Blood samples were collected in
preservative-free heparin. PBMC were obtained following Ficoll-Hypaque density gradient sedimentation and were analyzed by direct or indirect immunofluorescence for reactivity with a series of monoclonal antibodies using standard techniques. Cells were analyzed for reactivity with a panel of monoclonal antibodies: T3 (CD3), T4 (CD4), T8 (CD8), and NKH1 (CD56) (Coulter Immunology, Hialeah, FL). Immunofluorescence reactivity was determined by automated flow cytometry analyzing 10^4 cells in each sample (EPICS-C; Coulter Electronics, Hialeah, FL).

Cytoxicity assays. IL-12 was kindly provided by Genetics Institute (Cambridge, MA) as a purified recombinant protein with a specific activity of 5.2 x 10^6 U/mg protein. Purified recombinant IL-2 was graciously provided by Amgen (Thousand Oaks, CA). Its specific activity was 1.05 x 10^7 U/mg protein. Cytotoxicity assays were performed using cryopreserved cells from the group of patients with metastatic cancer and were compared with cryopreserved samples from healthy volunteers. In the cohort of patients who had undergone allogeneic BMT, assays were performed on fresh samples. For these experiments, PBMC freshly isolated from healthy volunteers served as controls. PBMC were evaluated for their ability to lyse NK-sensitive (K562) and NK-resistant (COLO 205) tumor cell targets. Effector cells were incubated for 18 hours in media alone (RPMI 1640 with 10% heat-inactivated human AB serum, 2% glucose, 1% penicillin-streptomycin, and 1% sodium pyruvate), media enriched with IL-2 (500 U/mL, 3 nmol/L), media enriched with IL-12 (10 U/mL, 35 pmol/L), or media containing both cytokines at the aforementioned concentrations. In selected patients, a range of concentrations of IL-2 (30 pmol/L to 30 nmol/L) and IL-12 (0.35 to 350 pmol/L) were used to assess dose-response. We found 35 pmol/L to be the optimal concentration for IL-12 and 3 nmol/L to be optimal for IL-2 in this setting, and therefore performed the remainder of our assays at these concentrations.

Proliferation studies. Cryopreserved PBMC were thawed and placed in 96-well round-bottom plates (Costar, Cambridge, MA) at a concentration of 2.5 x 10^5 cells/mL in a final volume of 200 µL. The cells were incubated with media alone or a combination of antibodies to the CD2 surface structure (anti-T3 plus anti-T4) at a concentration of 1:200. An aliquot of cells was also incubated in wells coated with antibody to the CD3 surface antigen (anti-T3 2ad2). Media, IL-2 (100 U/mL, 625 pmol/L), IL-12 (10 U/mL, 35 pmol/L), or a combination of the two cytokines was then added to the wells containing PBMC and each mitogenic stimulus. Samples were set up in triplicate. Plates were incubated for 96 hours at 37°C and then pulsed for 18 hours with 0.5 µCi [3H]-thymidine. Cells were then harvested, and [3H]-thymidine incorporation was determined by scintillation spectrometry.

Statistical analysis. Descriptive statistics are reported as proportions, medians, and means with standard errors.

RESULTS

Effect of IL-12 on cytotoxic function of PBMC from patients with metastatic solid tumor. PBMC were obtained from 12 patients with metastatic solid tumors. Their median age was 52 years (range, 23 to 70). There were seven men and five women. Diagnoses included melanoma (five patients), renal cell carcinoma (four patients), adenocarcinoma of the colon (one patient), non-small-cell lung cancer (one patient), and testicular cancer (one patient). Five of 12 patients had received prior cytotoxic chemotherapy. None had received any chemotherapy or radiotherapy for at least 8 weeks before PBMC were collected.

The immunophenotypic profile of PBMC from these cancer patients reflected a relatively normal distribution of CD4+ T cells, CD8+ T cells, and NK cells. The mean fraction of PBMC that expressed CD3 was 65% ± 6%; 49% ± 9% of cells expressed CD4 and 19% ± 4% expressed CD8. CD56+ NK cells accounted for 15% ± 3% of PBMC.

NK cytolytic activity, as measured by the ability to lyse K562 cells in a 4-hour chromium-release assay, was clearly diminished in these cancer patients compared with normal controls. Figure 1 shows that overnight incubation with IL-12 (35 pmol/L) dramatically increased cytolytic activity of PBMC from patients and restored NK activity to the level observed in PBMC from normal individuals. The augmentation of cytolytic activity with 35 pmol/L of IL-12 was almost equivalent to that noted when PBMC from patients were exposed overnight to 3 nmol/L of IL-2 (Fig 2A). Cytotoxic activity increased even further when cells were incubated with IL-2 and IL-12 simultaneously. In contrast to the observed effect on NK killing, overnight exposure of cryopreserved resting PBMC from patients with metastatic cancer to IL-12 did not markedly increase cytolytic activity against an NK-resistant target (COLO 205) (Fig 2B).

Effect of IL-12 on cytolytic activity of PBMC from patients receiving IL-2 in vivo. Six of 12 patients whose PBMC were assayed were entered onto an experimental study in which they received low-dose recombinant IL-2 (Hofmann-LaRoche, Nutley, NJ) by continuous infusion at a dose of 4.5 x 10^7 U/m2/d. We have previously reported that such treatment results in the selective expansion of circulating NK cells without any change in the number of T lympho-

![Fig 1. IL-12 restores NK activity of PBMC from patients with metastatic cancer. Cryopreserved PBMC from 12 patients with metastatic solid tumors exhibit a profound defect in their ability to kill NK-sensitive (K562) cells in a 4-hour 51Cr-release assay compared with cryopreserved PBMC from normal volunteers. Overnight incubation with IL-12 (10 U/mL, ~35 pmol/L) corrected this abnormality. (●) Controls; (□) patients.](image-url)
Fig 2. Comparative effect of IL-12 and IL-2 on NK activity of PBMC from patients with metastatic cancer. Cryopreserved PBMC from 12 patients were incubated overnight with either media alone (●), IL-12 (10 U/mL) (■), IL-2 (500 U/mL) (□), or a combination of IL-12 and IL-2 (○). The ability to lyse (A) NK-sensitive (K562) and (B) NK-resistant (COLO 205) cells in a 4-hour ^51Cr-release assay is displayed over a range of E:T ratios.

Fig 3. Effect of IL-12 on cytolytic activity of PBMC from patients receiving low-dose IL-2 in vivo for metastatic cancer. PBMC from six patients who were participating in a trial of low-dose IL-2 (4.5 × 10^6 U/m^2/d) were evaluated for their ability to kill (A) NK-sensitive (K562) and (B) NK-resistant (COLO 205) targets in a 4-hour ^51Cr-release assay. Cryopreserved PBMC were thawed and incubated in either media alone, IL-12 (10 U/mL), or IL-12 (500 U/mL). Cryopreserved PBMC obtained before initiation of therapy and after 4 to 5 weeks of continuous treatment were evaluated. (●) Pre IL-2 therapy; (■) on IL-2 therapy.
treatment was equivalent to that noted when these PBMC were incubated with additional IL-2 (500 U/mL, 3 nmol/L) in vitro.

Enhancement of cytolytic activity by IL-12 in PBMC obtained after allogeneic BMT. We also evaluated the effect of IL-12 on cytotoxicity in PBMC obtained from patients who had undergone allogeneic BMT. Thirteen patients with hematologic malignancies who had received high-dose cyclophosphamide, 1,400 cGy total-body irradiation, and HLA-compatible sibling allogeneic marrow that had been depleted of T cells with anti-T12 (CD6) and complement were studied. This group of patients comprised nine men and four women with a median age of 38 years (range, 21 to 53). Diagnoses included acute myelogenous leukemia (AML; three patients), acute lymphoblastic leukemia (ALL; three patients), chronic myelogenous leukemia (CML; three patients), multiple myeloma (MM; two patients), and non-Hodgkin's lymphoma (NHL; two patients). Mononuclear cells were obtained from these patients at a median of 30 days post-BMT (range, 19 to 54 days). The immunophenotypic profile of PBMC from these patients was similar to what we have observed in patients at a comparable stage after receiving CD6-depleted allogeneic marrow. CD3+ cells comprised 47% ± 5% of PBMC, whereas 42% ± 8% of cells were CD56+ NK cells. A total of 34% ± 4% of PBMC expressed CD8, while only 15% ± 3% expressed CD4.

The addition of IL-12 to PBMC from these allogeneic transplant recipients resulted in a marked increase in cytosis of both NK-sensitive and NK-resistant targets compared with cells incubated in media alone. The extent of NK killing induced by picomolar concentrations of IL-12 was comparable to that produced by nanomolar concentrations of IL-2 (Fig 4A). The degree of cytosis of NK-resistant COLO 205 targets, although enhanced by IL-12, was not as great as that produced by IL-2 at the concentration tested (Fig 4B). The addition of IL-12 to IL-2 did not appreciably increase in vitro cytolytic activity in marrow transplant recipients above that seen with either cytokine alone (data not shown).

Effect of IL-12 on proliferation of PBMC from patients with malignant disease. Although IL-12 by itself does not induce detectable proliferation of resting normal PBMC, it has been shown to enhance the proliferative signals delivered by several T-cell mitogens. The effect of IL-12 on proliferation of PBMC from five patients with metastatic cancer was evaluated. As shown in Fig 5, IL-12 by itself had no effect on 3H-thymidine incorporation. However, IL-12 did significantly increase 3H-thymidine incorporation in PBMC stimulated either through the T-cell receptor complex (with an anti-CD3 monoclonal antibody) or via an alternative pathway of T-cell activation (with anti-CD2 monoclonal antibodies). Nevertheless, IL-12 was less effective than IL-2 in enhancing proliferation of PBMC at the concentrations tested. Overall, mitogen-induced stimulation of DNA synthesis in the presence or absence of either IL-12 or IL-2 was inferior in cancer patients compared with healthy volunteers (data not shown).

DISCUSSION

The role immunoregulatory cells play in the development and progression of malignant disease remains poorly understood. However, it is clear that patients with metastatic cancer have demonstrable defects in both clinical and laboratory parameters of immune competence. Cytotoxic function, specifically NK lysis of K562 targets by PBMC, diminishes as many diseases progress. Moreover, several
studies have identified low NK-cell cytotoxic activity as an important prognostic factor for relapse in patients with both solid and hematologic neoplasms.\textsuperscript{32,33} Our current data confirm that PBMC from patients with metastatic cancer have a marked diminution in NK cytolytic activity compared with normal subjects, despite having a normal proportion of NK cells. We found that incubation of PBMC from these cancer patients with IL-12 could correct this defect. The influence of IL-12 on cytolytic activity was similar to that of IL-2. Notably, improvement in NK activity was observed when IL-12 was used at a concentration 2 log lower than the optimal concentration of IL-2. This difference in potency may have important clinical implications, as IL-2 treatment of malignant disease has often been limited by severe dose-related toxicities. If IL-12 is found to have antitumor activity in clinical trials at relatively low concentrations, the adverse side effects associated with such treatment may be less than those observed with IL-2.

Furthermore, our data suggest that IL-12 could serve as a potential adjunct to IL-2 for immunotherapy. Although IL-12 has been reported to enhance cytolytic activity against colon carcinoma targets when incubated with cells from healthy volunteers,\textsuperscript{34} we found that IL-12 by itself was unable to induce a marked increase in cytolytic activity against NK-resistant targets in patients with metastatic cancer. In addition, IL-12 had a limited capacity to expand the number of cytotoxic effector cells. Indeed, IL-12 has been shown to have little proliferative effect on resting NK cells or T cells, although IL-12 can support mitogen-stimulated T cells.\textsuperscript{20,21,35,36} In contrast, IL-2 provides a powerful proliferative stimulus to both NK cells and activated T lymphocytes.\textsuperscript{37,38} We have previously demonstrated in clinical trials that prolonged infusion of low doses of IL-2, sufficient to saturate only its high-affinity receptor, can dramatically expand the number of NK cells in patients with metastatic cancer with minimal toxicity.\textsuperscript{26} Although markedly increasing the number of circulating NK cells, IL-2 delivered at such low doses only modestly increases cytolytic activity against NK-resistant targets. However, a dramatic increment in cytotoxicity against NK-resistant targets is observed when these expanded NK cells are exposed to picomolar concentrations of IL-12 that, before IL-2 therapy, had little influence on cytolytic activity. Pretreatment with IL-2 may make PBMC more responsive to IL-12 because of upregulation of IL-12 receptors.\textsuperscript{36} Thus, one could envision the design of clinical trials in which the administration of IL-12 would be used to activate circulating cytotoxic effectors that had been expanded previously in vivo by low-dose IL-2.

BMT is another area in which IL-12 merits clinical exploration. Considerable clinical and experimental evidence support the notion that there exist immune mechanisms that can help to eliminate minimal residual disease after BMT.\textsuperscript{39,40} Both T lymphocytes and NK cells have been proposed as mediators of this graft-versus-leukemia (GVL) effect.\textsuperscript{23,41,42} Animal and human studies have suggested that IL-2 can induce GVL activity post-BMT.\textsuperscript{34,47} A number of clinical studies investigating the use of IL-2 in this setting have recently been published.\textsuperscript{16-18} Our current data suggest that PBMC from patients early post-BMT are responsive to IL-12, as well as to IL-2. The role of IL-12 in the induction of GVL activity remains to be determined.

The mechanism of action of IL-12 has not been fully elucidated.\textsuperscript{20,21} IL-12 appears to act via a mechanism that is independent of IL-2. We have previously demonstrated that the effects of IL-12 on NK cells are not blocked by monoclonal antibodies to the IL-2 receptor.\textsuperscript{23} In addition, unlike the activity of IL-2, the activity of IL-12 is resistant to inhibition by IL-4. However, like IL-2, IL-12 can stimulate production of certain cytokines (eg, interferon-\gamma and tumor necrosis factor).\textsuperscript{46} Its effects on the elaboration of other cytokines is currently under investigation.

The current report suggests that IL-12 can enhance cytolytic activity of PBMC from patients with malignant disease. The precise role that IL-12 will play clinically remains unknown. Its toxicity spectrum needs to be defined in animal and human studies. However, it is hoped that IL-12 will be useful in inducing cytotoxicity against tumors in the clinical arena and that it will be a valuable addition to IL-2 in future immunotherapeutic trials.

\section*{Acknowledgment}

The authors thank Dr Steven Herrmann, Genetics Institute, for providing IL-12, and Patrice Noonan for assistance in the preparation of the manuscript.

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RJ Soiffer, MJ Robertson, C Murray, K Cochran and J Ritz