ABSTRACT

Systemic interleukin 2 (IL-2) and IL-2-activated lymphocytes have induced tumor regression in some cancer patients. The IL-2-activated cells have usually been generated by obtaining peripheral blood mononuclear cells (PBMC) from cancer patients shortly after systemic IL-2 therapy and culturing them with IL-2 in vitro. In an effort to augment the ex vivo generation of such cells preactivated in vivo, we examined the proliferative responses of PBMC from IL-2-treated cancer patients to several proliferative signals including IL-2, interleukin 4 (IL-4), and mitogenic antibodies to CD3 and CD28. Although much is known about the response of normal PBMC to these signals, the possibility was considered that the response of lymphocytes preactivated by IL-2 in vitro might differ from that of normal PBMC. Accordingly, PBMC obtained from ten normal, healthy controls and from 17 patients with advanced cancer 1 to 3 days after systemic IL-2 therapy were cultured for 4 days with IL-4 (1000 units/ml) and/or IL-2 (10 units/ml or 1000 units/ml) or with combinations of IL-4 and anti-CD3 ± anti-CD28, and they were then tested for proliferation by [3H]Thymidine incorporation. IL-4 failed to induce proliferation of normal PBMC and inhibited IL-2-induced proliferation, whereas IL-4 alone induced proliferation in PBMC from five of 11 IL-2-treated patients and did not inhibit but augmented the proliferation induced by IL-2 (10 units/ml and 1000 units/ml) in PBMC from six of nine patients and five of 11 patients, respectively. Anti-CD3 induced proliferation in PBMC from eight of nine patients, and the proliferation was consistently augmented by coculture with anti-CD28. Finally, IL-4 significantly augmented the proliferative responses of PBMC from IL-2-treated patients to anti-CD3, as well as to the combination of anti-CD3 and anti-CD28. Thus, in PBMC from IL-2-treated cancer patients, IL-4 enhanced the in vitro proliferation induced by IL-2 or by anti-CD3 ± anti-CD28. The results suggest that IL-4 and/or mitogenic antibodies may be useful in augmenting the ex vivo generation of lymphocytes for clinical adoptive immunotherapy.

INTRODUCTION

The systemic administration of IL-2 and autologous lymphocytes activated by culture with IL-2 has induced tumor regression in some cancer patients (1–3). However, the responses have been observed in only a minority of patients, and the doses of IL-2 required have usually been toxic (4, 5). In animal models, the antitumor effects observed correlated with the amount of IL-2 administered and with the number of activated cells infused (6). Thus, the development of techniques to augment the generation of activated lymphocytes ex vivo may potentially enhance tumor responses in vivo. One approach to this goal is the use of other proliferative signals in addition to or in lieu of IL-2.

IL-2-activated cells for therapy are usually generated by obtaining PBMC from cancer patients shortly after systemic IL-2 therapy and culturing them with IL-2 in vitro. The cells thus generated for infusion are phenotypically and functionally heterogeneous and contain cells with LAK activity (5, 7), as well as other cells which may mediate or contribute to antitumor responses (8). In an effort to enhance the generation of such cells in vitro, several agents which induce a proliferative response in normal human PBMC were studied for their possible proliferative effects on PBMC from IL-2-treated patients.

IL-4 is a multipotent lymphokine which enhances proliferation of B-cells, T-cells, natural killer cells, and monocytes (9–17). In normal lymphocytes, IL-4 is comitogenic with antigen (15), lectins (16, 17), and antibodies to CD3 (17). IL-4 can augment the generation of antigen-specific cytotoxic T-cells (18–20) and can induce non-major histocompatibility complex-restricted LAK activity in human PBMC preactivated with IL-2 (21, 22). In contrast to the pleotropic effects of IL-4, monoclonal antibodies directed against CD3 (anti-CD3) specifically activate and induce the proliferation of CD3+ T-cells (23) and can support the growth of major histocompatibility complex-restricted T-cells (24) as well as cells with LAK activity (25). The proliferative response to anti-CD3 can be enhanced using monoclonal antibodies directed against CD28, a 44,000 homodimer found on approximately 80% of peripheral T-cells (26). The proliferative response of normal lymphocytes to anti-CD3 is also known to be enhanced by IL-4.

Although much is known about the proliferative response of normal lymphocytes to these agents, the response of lymphocytes from IL-2-treated patients, i.e., cells which have been preactivated in vivo by IL-2, and, potentially, by other intermediary lymphokines and cytokines, may be different from that of normal lymphocytes. For example, IL-4 inhibits IL-2-induced LAK activity in normal human PBMC, but enhances IL-2-induced LAK activity in PBMC preactivated by IL-2 (21, 22). Accordingly, the proliferative effects of IL-4 and mitogenic antibodies on PBMC from IL-2-treated cancer patients were examined. The data demonstrate that (a) in contrast to the inhibition of IL-2-induced proliferation by IL-4 in normal PBMC, IL-4 alone induced substantial proliferation and augmented the proliferative response to IL-2 in PBMC from the majority of the patients tested; (b) anti-CD3 induced proliferation of patient PBMC; (c) anti-CD28 enhanced the proliferative response to anti-CD3; and (d) IL-4 augmented the proliferative response to both mitogenic antibodies. The results suggest that IL-4 ± mitogenic antibodies may have a role in the ex vivo generation of lymphocytes for adoptive immunotherapy trials.

MATERIALS AND METHODS

Study Population and Blood Sampling. Seventeen patients aged 30 to 71 yr with metastatic renal cell carcinoma (9 patients), melanoma (6 patients), diffuse histiocytic lymphoma (one patient), and carcinoma of the colon (one patient) participated in a Phase I/II trial of IL-2 and adoptive transfer of LAK cells (3, 4). Patients had no preceding treatments within 4 wk of study and had Karnofsky scores ≥70% at initiation...
of IL-2 therapy. All patients received systemic recombinant human IL-2 (Hoffman-LaRoche Inc., Nutley, NJ), with treatment cycles consisting of IL-2 administered by continuous i.v. infusion for 5 consecutive days. Seven patients received 1 to 4 treatment cycles of IL-2 (3 × 10^8 units/m²/day) (4, 5), and 10 patients received a single cycle of IL-2 at a higher dose (6 × 10^8 units/m²/day) (3). Blood samples were collected 1 to 3 days after termination of a treatment cycle, which corresponded to the time of initial leukapheresis for ex vivo cell culture for LAK generation. Blood was collected from healthy controls on the day of assay.

Preparation of Human Blood Mononuclear Cells and T-Cells. PBMC were isolated from heparinized peripheral blood by Ficoll-Hypaque density gradient separation. For purification of T-cells, PBMC were adherence depleted of macrophages followed by rosetting with 2-amino-ethyl-isothiouronium bromide-treated sheep RBC (27). The resultant population was >90% CD3+ and <0.5% Mo3+ as assessed by fluorescence-activated cell sorting.

Lymphokines. Purified recombinant human IL-4 was kindly provided by Immunex Corp., Seattle, WA, with a specific activity of 10^6 units/mg as measured in a cytotoxic T-lymphocyte line (CTL) assay.

Monoclonal Antibodies. Murine monoclonal antibodies 64.1 (IgG2a anti-CD3) and 9.3 (IgG2a anti-CD28) have been described previously (26). Antibodies purified from ascites fluid by Protein A-Sepharose affinity chromatography were kindly provided by Dr. Paul Martin, Fred Hutchinson Cancer Research Center, Seattle, WA.

Lymphocyte Proliferation Assays. Cells were cultured at 37°C in complete medium consisting of RPMI 1640 supplemented with penicillin, streptomycin, L-glutamine, and heat-inactivated human AB serum (15%). In the proliferation assays, PBMC or purified T-cells were cultured in triplicate for 4 days at 2.5 × 10^5 cells/well in 96-well flat-bottomed culture plates with various combinations of IL-2, IL-4, anti-CD3, and anti-CD28. Anti-CD3 and anti-CD28 were used in soluble form except with purified T-cells, in which anti-CD3 was immobilized onto the wells of flat-bottomed plates (29). Six to 8 h prior to harvesting, 1 μCi of [3H]dThd (6.7 Ci/mM specific activity; New England Nuclear Corp., Boston, MA) was added to cultures. Cells were harvested onto glass fiber filters, and [3H]dThd incorporation was quantified by liquid scintillation counting. Criteria used to define proliferation or the enhancement of proliferation were (experimental cpm - control cpm) > 500 cpm.

In the experiments involving in vitro IL-2 preactivation, PBMC were cultured with IL-2 (250 units/ml ± 2.5 × 10^5 cells/ml) in 24-well culture plates. After 4 days, the cells were harvested, washed extensively with phosphate-buffered saline, and then further cultured in proliferation assays as described above.

RESULTS

Augmentation by IL-4 of IL-2-driven Proliferation of PBMC from IL-2-treated Patients. To determine the proliferative response to IL-4 of PBMC preactivated by the systemic administration of IL-2 in vivo, PBMC were obtained from patients 1 to 3 days after IL-2 therapy, were cultured for 4 days with IL-4 (1000 units/ml) alone or with a combination of IL-4 (1000 units/ml) plus IL-2 (10 units/ml or 1000 units/ml), and were then tested for [3H]dThd incorporation. The time for obtaining PBMC from patients was selected on the basis of the observation that the 1 to 3 days after completing IL-2 therapy represents the time of maximal rebound lymphocytosis and expression of activation markers on PBMC (5, 30), and the time when patient PBMC are usually obtained by leukapheresis for ex vivo LAK cell generation for infusion. The lower concentration of IL-2 which induces suboptimal proliferation was used to facilitate detection of enhancing or inhibitory effects of IL-4. The effect of IL-4 on proliferation induced by the high concentration of IL-2, which is normally used for LAK cell generation in clinical trials (1-3), was also studied. The results are shown in Table 1. IL-4 alone failed to induce proliferation and inhibited IL-2-driven proliferation in PBMC from healthy controls (n = 7), as previously reported (16, 22, 31). By contrast, IL-4 induced proliferation in PBMC from 5 of 11 IL-2-treated patients. Furthermore, rather than inhibit proliferation induced by IL-2, IL-4 augmented proliferation induced by IL-2 (10 units/ml or 1000 units/ml) in PBMC from 6 of 9 and 5 of 11 IL-2-treated patients, respectively.

Dependence of the Proliferative Effects of IL-4 upon Preactivation of Lymphocytes by IL-2 in Vivo or in Vitro. To determine whether the ability of IL-4 to augment IL-2-induced proliferation in PBMC from IL-2-treated cancer patients reflected the effects of systemically administered IL-2 or the presence of cancer, PBMC were obtained from a patient with metastatic melanoma before and after IL-2 therapy and cultured in IL-4 (1000 units/ml) ± IL-2 (1000 units/ml). The results are presented in Table 2. As in normal PBMC, IL-4 inhibited IL-2-induced proliferation of patient PBMC before IL-2 therapy, whereas in PBMC obtained 24 h after therapy, IL-4 did not inhibit, but rather augmented IL-2-induced proliferation. Thus, the altered proliferative response of the PBMC to IL-4 appeared to depend upon systemic therapy with IL-2.

The effects of systemic IL-2 may be secondary to the secretion of intermediary lymphokines or cytokines in vivo. To determine whether the proliferative effects of IL-4 on PBMC preactivated with IL-2 in vivo would be similar to those of cells preactivated in vitro, PBMC from normal healthy individuals were preincubated with IL-2 (250 units/ml) for 4 days and then tested for a proliferative response to IL-4, IL-2, and to a combination of IL-2 and IL-4. The results, presented in Table 3, demonstrate that IL-4 induced proliferation and augmented the proliferation induced by IL-2 (10 units/ml) in cells preincubated with IL-2 but not in fresh PBMC.

Anti-CD3 Induction of Proliferation of PBMC from IL-2-treated Patients and Anti-CD28 Enhancement of This Response. Anti-CD3 is mitogenic in normal PBMC (23), and anti-CD28 augments that response (26). However, systemic IL-2 has a variety of immunomodulatory effects, and the response of lymphocytes from IL-2-treated patients to mitogenic signals may differ from that of normal lymphocytes (32). To determine the proliferative response to anti-CD3 (1 μg/ml) ± anti-CD28 (2 μg/ml) of PBMC obtained from patients after IL-2 therapy, PBMC obtained from patients after IL-2 therapy and from healthy controls were cultured for 4 days with anti-CD3 ± anti-CD28 and tested for proliferation. The results are shown in Table 4. Anti-CD3 induced proliferation in both PBMC from 6 of 6 normal controls in 13 of 13 experiments and in PBMC from 8 of 9 IL-2-treated patients in 8 of 9 experiments. However, the responses to anti-CD3 of PBMC from patients (median cpm = 2700) were significantly lower than that by PBMC from controls (median cpm = 16399, P < 0.05 by rank-sums test). Anti-CD28 augmented the proliferative response to anti-CD3 in PBMC from 4 of 4 healthy controls and in PBMC from 5 of 5 IL-2-treated patients.

Comitogenic Effect of IL-4 and Anti-CD3 ± Anti-CD28 upon PBMC from IL-2-treated Patients. IL-4 is a potent costimulator of proliferation when used in combination with mitogens or anti-CD3 in normal PBMC (14-17). To determine its effect on PBMC preactivated by IL2, PBMC from IL-2-treated patients were cultured with combinations of IL-4 (1000 units/ml), anti-CD3 (1 μg/ml), and anti-CD28 (2 μg/ml) and tested for proliferation. The results are shown in Table 5. IL-4 significantly and, in some cases, dramatically augmented anti-CD3-induced
proliferation of PBMC from 7 of 8 patients. IL-4 further augmented the proliferative responses to the combination of anti-CD3 and anti-CD28 in PBMC from 5 of 6 patients tested.

**Induction of Proliferation by IL-4 and Anti-CD3 of T-Cells from IL-2-treated Patients.** Although anti-CD3 specifically stimulates the proliferation of T-cells, IL-4 can affect a variety of cell lineages, including macrophages (12) and non-T-cells (9–11, 13). Therefore the mechanism by which IL-4 augments the proliferative response of PBMC from IL-2-treated patients may include effects on accessory cells, which are important in anti-CD3-induced proliferation (23). To characterize the cells proliferating in response to IL-4 and anti-CD3, the proliferative response and accessory cell dependence of purified T-cells from IL-2-treated patients in response to IL-4 and anti-CD3 were examined. T-cells were purified by erythrocyte rosetting (27), cultured with IL-4 (1000 units/ml) and anti-CD3 (1 μg/ml or immobilized) for 4 days, and then tested for proliferation. The results are shown in Table 6. As reported with normal T-cells (23), T-cells preactivated by IL-2 in vivo did not proliferate in response to soluble anti-CD3, but the addition of plastic-adherent accessory cells at the beginning of culture restored the response. By contrast, T-cells exhibited proliferation in response to anti-CD3 immobilized onto plastic dishes, and IL-4 significantly augmented proliferation without a requirement for accessory cells. Thus, IL-4 and anti-CD3 induced proliferation of purified T-cells from PBMC preactivated by IL-2 in vivo, and the effect of IL-4 was not strictly accessory cell dependent.

**DISCUSSION**

A potential way to improve the efficacy of systemic IL-2 therapy of human cancer is to develop techniques to augment
The ex vivo generation of activated lymphocytes for adoptive transfer (6). Lymphocytes for therapy have been generated by culturing PBMC obtained from cancer patients shortly after systemic IL-2 therapy with IL-2 in vitro. IL-2 administration produces a rebound lymphocytosis (5, 30), which allows for a higher recovery of cells for ex vivo culture. Systemic IL-2 also activates PBMC in vivo, with the increased expression of activation markers HLA-DR and TaC (p55 IL-2 receptor) on activated PBMC in vivo, with the increased expression of activation markers (5). In an effort to augment the ex vivo generation of activated cells, we have examined the proliferative response of PBMC from IL-2-treated patients, i.e., cells preactivated by IL-2 in vivo, to IL-4, anti-CD3, and anti-CD28. IL-4 inhibits the proliferative response of normal PBMC to IL-2, but augments the proliferation of lymphocytes preactivated by IL-2 in vitro (22). Therefore, it was hypothesized that IL-4 might augment IL-2-induced proliferation in PBMC from IL-2-treated patients, i.e., PBMC preactivated with IL-2 in vivo. The results demonstrate that, in PBMC from IL-2-treated patients, IL-4 induces proliferation and augments IL-2-driven proliferation. In addition, anti-CD3 induced proliferation of PBMC from IL-2-treated patients which proliferation was enhanced by anti-CD28, and IL-4 augmented the proliferation induced by these mitogenic antibodies.

The mechanism by which IL-2 therapy alters the proliferative response of PBMC to IL-4 is not known. IL-4 induces the proliferation of lymphocytes activated by antigen or mitogen (15-17) or by preincubation with IL-2 in vitro (22). Thus, systemic IL-2 and/or other intermediary cytokines produced endogenously in response to systemic IL-2 (30) are likely to result in preactivation of PBMC, including the observed increase in IL-2 receptor expression (5). IL-4 appears to down-regulate the responsiveness of normal, resting lymphocytes to IL-2, possibly by inhibiting the increase in IL-2 receptor expression (22). By contrast, IL-4 does not inhibit, but augments proliferation of PBMC preactivated by IL-2 which already have increased expression of IL-2 receptors. IL-4 also enhances the proliferation induced by mitogens which may directly enhance the expression of IL-2 receptors. Thus, IL-4 may represent a regulatory factor which promotes the proliferation of activated cells, but inhibits IL-2-induced proliferation of bystander cells by inhibiting IL-2 receptor expression.

The proliferative response of PBMC from IL-2-treated patients to anti-CD3 appeared to be less than that of PBMC from healthy controls. This is unlikely to be due to a decrease in circulating T-cells, since previous studies have shown that, after continuous-infusion IL-2 therapy, there is a rebound expansion of peripheral T-cells (5, 30) with no significant change in the percentage of CD3+, CD4+, or CD8+ T-cells (5). Since the lymphocyte proliferation induced by anti-CD3 is mediated by autocrine lymphokine production (23), the decreased proliferative response observed might be a function of either decreased lymphokine production or decreased lymphokine receptor expression. No evidence for either hypothesis has yet been obtained.

Although IL-4 induces little or no proliferation in normal, resting PBMC (16), IL-4 augments the proliferation of T-cells costimulated by antigen (15), mitogen (16, 17), or antibodies to the CD3 molecular complex (17). Similarly, IL-4 enhanced the proliferation of PBMC from IL-2-treated patients costimulated with anti-CD3. The enhancement of proliferation of normal PBMC by IL-4 appears to be mediated by both IL-2-dependent and IL-2-independent pathways. Antibodies to the p55 IL-2 receptor (anti-Tac) inhibit augmentation of anti-CD3-induced proliferation by IL-4 (17), suggesting that costimulation by IL-4 may increase autocrine IL-2 production. However, anti-Tac does not completely block the augmentation, and IL-4 augments the proliferation of T-cells in the presence of cyclosporin A which blocks endogenous IL-2 production (17), suggesting that IL-4 can act in an IL-2-independent manner. Our observation that IL-4 could augment the proliferation of IL-2-treated PBMC induced by an optimal concentration of IL-2 (1000 units/ml) is consistent with the hypothesis that IL-4 does more than increase endogenous IL-2 production. IL-4 can augment the expression of the p55 IL-2 receptor induced by low concentrations of IL-2 (1 unit/ml) in human PBMC pretreated by IL-2 in vitro (22). Thus, the enhancement of anti-CD3-induced proliferation by IL-4 of PBMC preactivated by IL-2 in vivo may reflect an upregulation of IL-2 receptor expression.

Table 6 Proliferation of T-cells from IL-2-treated patients in response to IL-4 and anti-CD3

<table>
<thead>
<tr>
<th></th>
<th>Soluble anti-CD3</th>
<th>Immobilized anti-CD3</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
<td>No IL-4</td>
</tr>
<tr>
<td>PBMC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purified T-cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>177</td>
<td>8,053</td>
<td>22,589</td>
</tr>
<tr>
<td>214</td>
<td>376</td>
<td>3,219</td>
</tr>
<tr>
<td>223</td>
<td>6,131</td>
<td>18,160</td>
</tr>
</tbody>
</table>

* AC, adherent cells added to purified T-cells to serve as accessory cells.

CD28 is a M, 44,000 cell surface homodimer present on approximately 80% of T-cells (26). The natural ligand for CD28 is not known, but monoclonal antibodies to CD28, although not mitogenic alone, can function as second signals to induce increased IL-2 production, IL-2 receptor expression, and proliferation of T-cells which have been primarily activated by anti-CD3 (26). Similarly, anti-CD28 augmented the proliferation of PBMC from IL-2-treated patients induced by anti-CD3. In addition, the combination of anti-CD3, anti-CD28, and IL-4 further enhanced proliferation. Thus, although anti-CD28 is not a primary activation signal, it may be useful for further enhancing cell growth.

PBMC represent a heterogeneous cell population, including both T-cell and non-T-cell populations. Because IL-4 is multipotent, with effects on a variety of hematopoietic cell lineages (9-17), its observed effects may be on both T- and non-T-cell populations. Similarly, anti-CD3, although a specific T-cell mitogen, may potentially induce proliferation of non-T-cells through the release of cytokines. Since accessory cells are important in the proliferative response of lymphocytes to anti-CD3 (23), one potential mechanism by which IL-4 might augment the proliferative response to anti-CD3 might be by enhancing accessory cell function. However, IL-4 augmented anti-CD3 in the absence of accessory cells when immobilized anti-CD3 was used, indicating that the effects of IL-4 were more directly on T-cells. Thus, the proliferative effects of IL-4 and anti-CD3 appear to have been on the T-cell fraction of PBMC preactivated by IL-2 in vivo, and the enhancement of proliferation by IL-4 was a direct effect of IL-4 on T-cells.

The proliferative effects of IL-4 alone and in combination with anti-CD3 ± anti-CD28 suggest a potential use of these agents for augmenting the generation of ex vivo activated cells for cancer therapy. IL-4 has recently been shown to induce LAK activity in PBMC preactivated by IL-2 (21, 22), and thus it could potentially be used with or without anti-CD3 for LAK cell generation in short- or long-term culture (25). IL-4 may...
IL-4 ENHANCEMENT OF PROLIFERATION AFTER IL-2 THERAPY

also be useful for the ex vivo expansion of other potentially therapeutic cells (33). IL-4 augments the generation of specific cytotoxic T-cell responses (18–20), and IL-4 can support the growth and tumor-specific cytolytic activity of TIL cultured in IL-2 (34). The combination of IL-4 and anti-CD3 ± anti-CD28 could potentially increase the cell number and/or function of in vitro cultured TIL. Finally, since a limitation of systemic IL-2 therapy of cancer is dose-related toxicity (4, 5), the ability of IL-2 could potentially be exploited by using the systemic administration of IL-4 in combination with lower, less toxic doses of IL-2. Such studies must await the results of ongoing Phase Ib clinical trials with IL-4.

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Enhancement by Interleukin 4 of Interleukin 2- or Antibody-induced Proliferation of Lymphocytes from Interleukin 2-treated Cancer Patients

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