Evidence for Eosinophil Activation in Cancer Patients Receiving Recombinant Interleukin-4: Effects of Interleukin-4 Alone and following Interleukin-2 Administration


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ABSTRACT

Interleukin-4 (IL-4) is a T-cell-derived cytokine that may mediate murine tumor rejection through the activation of host eosinophils. In association with a Phase I clinical trial of IL-4 in cancer patients, we have examined changes in eosinophil counts and characterized systemic eosinophil degranulation. As previously reported, IL-4 administration induced a modest eosinophilia in all 17 evaluated patients. Here, we report that IL-4 therapy induced systemic eosinophil degranulation based on increases in serum major basic protein (MBP) (P = 0.018) and urine MBP (P = 0.031). The increase in serum MBP was IL-4 dose dependent (P = 0.001). Following the highest dose (600 pg/m2/day) of IL-4 administered, mean serum MBP levels were >2000 ng/ml. Skin biopsies of rashes from patients receiving IL-4 revealed MBP deposition. Sera from eight patients receiving IL-4 at 360 and 600 pg/m2/day exhibited eosinophil survival-enhancing activity (on days 3, 5, 7, and 9) significantly above pretreatment (on day 1) activity (P values 0.0469, 0.0039, 0.0395, and 0.0313, respectively). This enhanced eosinophil survival could be neutralized by antibodies to IL-5, granulocyte-macrophage-colony-stimulating factor, and IL-3. The eosinophil activation demonstrated in this trial may be relevant to the clinical effects of IL-4 in cancer patients. Furthermore, an association between IL-4 and eosinophil activation should be explored in other disease states.

INTRODUCTION

IL-4, a 20-kDa glycoprotein secreted by activated T lymphocytes, promotes the proliferation of resting B cells, enhances the activation of T cells, increases natural killer cell activity following exposure to IL-2, and induces the expression of MHC antigens, adhesion molecules (V-CAM), and IgE Fc receptors (CD23) on the surface of a variety of cells (1-6). An experimental system utilizing tumor cells transfected with an expression vector containing the cDNA for IL-4, allowing for constitutive production, has been designed to assess the role of IL-4 in murine tumor rejection (7). Tumor rejection accompanied by a significant local eosinophil infiltration was induced by the local expression of IL-4 in one such model (7). In vivo antibody-mediated depletion of eosinophils has been shown to block the antitumor effect of IL-4 in this tumor system (8).

Eosinophilia has been classically associated with several disease states, most notably allergic diseases and helminth infections (9). However, there is increasing evidence that eosinophils may be involved in a broader spectrum of diseases (9). Eosinophils are capable of killing a variety of targets in vitro, including tumor cells (10). They are also capable of secreting a number of different cytokines and several cytotoxic proteins, including MBP (9). In vitro, IL-5, IL-3, and GM-CSF can stimulate eosinophil production and enhance the function of mature eosinophils (11-13). Although IL-5 effects are specific for eosinophils, IL-3 and GM-CSF also promote the expansion of neutrophils and macrophages. In vivo, IL-5 plays a central role in eosinophilia associated with parasitic infections and IL-2 therapy (14-16). Administration of IL-3 or GM-CSF can induce a modest eosinophilia (17, 18).

In this clinical trial, we have characterized the effects of IL-4 administration alone and after a short course of IL-2 on peripheral eosinophil counts and systemic eosinophil activation. This report demonstrates that IL-4 therapy can lead to systemic eosinophil degranulation. Sera from patients receiving IL-4 were able to enhance eosinophil survival in vitro. The systemic activation of eosinophils could potentially play a role in immune-mediated tumor regression or cytokine-induced vascular toxicity.

MATERIALS AND METHODS

Study Design. All study patients were \(\geq\)18 years of age with histologically proven nonhematological cancer refractory to standard therapy, and their characteristics and eligibility requirements were previously described (19). Each patient received a continuous infusion of IL-4 administered for 7 consec-
Eosinophil Activation in Patients Receiving IL-4

Table 1 Treatment plan

<table>
<thead>
<tr>
<th>IL-4 dose levels</th>
<th>Week 1: IL-4 CI* for 7 days</th>
<th>Week 4: IL-2 CI for 4 days</th>
<th>Week 5: IL-4 CI for 7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 μg/m²/day*</td>
<td>6</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>120 μg/m²/day</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>360 μg/m²/day</td>
<td>5</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>600 μg/m²/day*</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* CI, continuous infusion.

** IL-2 dose for all patients = 11.2 MIU/m²/day CI.

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Eosinophil Survival Bioassay. Eosinophils were purified from heparinized peripheral blood of normal donors on a one-step Percoll gradient (d = 1.082) to remove mononuclear cells followed by hypotonic shock for erythrocyte lysis. The pelleted granulocytes were incubated with anti-CD16-conjugated magnetic microbeads. Anti-CD16-bound neutrophils were removed using a magnetic separation system (MACS; Miltenyi Biotec, Sunnyvale, CA; Ref. 22). Eosinophils were >95% pure and >98% viable. Fifty-μl aliquots of purified eosinophil preparations (2.0 × 10⁵ cells/ml) suspended in Hybricare medium (American Type Culture Collection, Rockville, MD) supplemented with 10% FCS were cultured with 10 μl patient serum or dilutions of recombinant human IL-5 (Schering-Plough Research Institute, Bloomfield, NJ) and 40 μl medium in 96-well flat-bottomed half-area plates (Costar, Cambridge, MA). Sera were obtained from patients before and during IL-4 therapy and was heat-inactivated prior to inclusion in the bioassay. All samples were tested in duplicate. After 4–5 days at 37°C and 5% CO₂, cells were stained with fluorescein diacetate, and cell counts were performed to determine cell recovery and viability. A comparison was made of the eosinophil survival response to patient serum samples, 100 pg/ml IL-5 preparations, and media alone. IL-4 alone has no effect on the eosinophil survival when added to the assay. All 17 study patients were screened on days 1–9 (initial IL-4 therapy) with this bioassay. Twelve of these patients were also screened during days 22–37 (IL-2 followed by second IL-4 infusion). Normal human sera and control media were comparable in their support of eosinophil survival.

Blocking of Bioactivity in Serum with Neutralizing Antibodies. The assay was performed by using the eosinophil survival bioassay described above with the additional step of preincubation of serum sample with rat mAbs to recombinant human IL-3, recombinant human IL-5, and recombinant human GM-CSF (gift from Dr. John Adams, DNAX, Palo Alto, CA) alone and in combination at a concentration of 1 μg/well for 1 h at room temperature prior to the addition of the eosinophils. The activity of these mAbs was reported previously (23). The neutralizing anticytokine antibodies remained present throughout the assays. These studies were performed on samples from days 1 to 9 (IL-4 infusion) on six patients (three at the 360-μg IL-4 dose, three at the 600-μg IL-4 dose) and on nine patients on days 22–26 (IL-2 infusion).

Statistical Analysis. Mean increase in eosinophil counts and serum and urine MBPs was examined using the paired t test. Differences in these variables across dose levels were examined by ANOVA. Two-sided α levels of 0.05 were considered statistically significant. No adjustments of the α levels for multiple testing were made. Eosinophil survival data were analyzed using the Wilcoxon signed rank test on the change from baseline (day 1) at days 3, 5, 7, 9, 22, and 26. One-tailed P values <0.05 were considered significant.

4 H. Kita, unpublished observations.
RESULTS
Changes in Peripheral Eosinophil Counts with IL-4 Therapy. Modest, but statistically significant \( P = 0.0004 \), increases in eosinophil counts were observed for all 17 patients combined with a mean increase of 368/μl and median increase of 256/μl (Fig. 1). In four patients, eosinophil increases ranged from 500 to 1000/μl (1–40 μg, 2–360 μg, and 1–600 μg), and for one patient (at the 360-μg dose) increases in eosinophil counts were >1000/μl. No clear IL-4 dose effect on eosinophil counts was discernible. Toxicity of IL-4 did not appear to correlate with the magnitude of the increases in eosinophil counts. However, 2 of 5 patients with eosinophil increases >500/μl after IL-4 therapy developed a skin rash, whereas only 1 of 12 patients with eosinophil increases <500/μl developed a rash.

Systemic Eosinophil Degranulation as Indicated by Increases in Serum and Urine MBPs. A significant increase in both serum MBP \( (P = 0.018) \) and urine MBP \( (P = 0.031) \) was demonstrated for all 16 evaluated patients. There was a strong correlation between the increase in serum MBP and urine MBP among the patients as demonstrated by a correlation coefficient of 0.758 with a \( P = 0.0007 \). As illustrated in Fig. 2, both the serum and urine MBP increases exhibited an IL-4 dose effect \( (P = 0.001 \) for serum MBP and \( P = 0.019 \) for urine MBP increase). Mean serum MBP increases ranged from -47 ng/ml at the 40-μg IL-4 dose to 1308 ng/ml at the 600-μg IL-4 dose level (Fig. 2A). Mean urine MBP increases ranged from 12 ng/ml at the 40-μg IL-4 dose to 233 ng/ml at the 600-μg IL-4 dose (Fig. 2B). In association with elevations in urine MBP, patients did not experience significant increases in leukocytes on urinalysis or significant proteinuria (≥2+). Two of the patients treated at the 600-μg/m² IL-4 dose had the highest serum MBP levels (1857 and 2525 ng/ml) at the conclusion of IL-4 therapy in association with a dose-limiting vascular leak syndrome (19).

Serum and urine MBP levels were markedly increased following the 4-day course of IL-2 (data not shown) as reported previously (15). Serum MBP levels ranged from 2801 to 8736 ng/ml following IL-2 treatment prior to the initiation of the second IL-4 infusion. Following the second IL-4 infusion, serum MBP levels declined to a variable degree for all patients.
evaluated. However, the mean serum MBP levels were greater at day 35 (following 6 days of IL-4 therapy) in those patients treated at 360 μg (4695 ng/ml) compared to the 120-μg (3204 ng/ml) and 40-μg IL-4 dose levels (3061 ng/ml).

**MBP Deposition in Skin Biopsies.** Skin biopsies from the proximal upper extremities were obtained from patients treated at the 360-μg and 600-μg IL-4 dose levels and were examined by standard hematoxylin and eosin histological evaluation and by immunohistochemical staining for deposition of MBP. As shown in Fig. 3, a patient developing a rash while receiving IL-4 had focal accumulation of eosinophils within the upper dermis on histological evaluation. Immunohistochemical
Clinical Findings. As previously reported, the 17 patients enrolled into the trial included 10 with renal cell cancer, 4 with melanoma, 2 with colon cancer, and 1 with cholangiocarcinoma (19). Fifteen patients had received either radiation, chemotherapy, or biological therapy prior to enrollment, and eight patients had a PS = 0 and 9 had a PS = 1. A single patient with metastatic renal cell cancer, who was treated at the lowest dose of IL-4 (40 μg/m²), demonstrated a minor response of lymph node disease lasting >30 months. This patient did demonstrate an increase in both urine MBP (from 17.0 to 41.1 ng/ml) and peripheral eosinophils (+576/µl) with IL-4 administration.

DISCUSSION

Therapy of cancer patients with various cytokines is based on the premise that these agents, such as IL-2, activate host lymphocytes to mediate either tumor-specific or nonspecific cytotoxic effects (24). IL-2 therapy is also associated with peripheral eosinophilia and the release of eosinophil proteins into patient’s serum, urine, and tissues due to the release of IL-5, presumably from activated T lymphocytes (15, 16, 25). More recently, other cytokines such as IL-4 have been studied in preclinical and clinical settings as possible antitumor agents. IL-4 is an important growth factor critical to the activation of a subset of T-helper cells (Th2) involved in antibody production (IgG1, IgE) and eosinophil activation (26). A murine tumor model utilizing transfection of tumor cells with IL-4 cDNA has demonstrated eosinophil infiltration at the site of rejecting tumor and in vivo depletion of eosinophils abrogates the antitumor effect (7, 8).

In this report, we have characterized the activation of eosinophils in cancer patients receiving IL-4 alone and IL-4 following a short course of IL-2. We have previously reported the clinical results of this Phase I trial in depth and briefly described the modest peripheral eosinophilia associated with IL-4 infusion (19). In the present report, we have shown that IL-4 therapy is not only associated with eosinophilia, but also eosinophil activation and degranulation within serum, urine, and skin based on the presence of MBP. Furthermore, sera from patients receiving IL-4 exhibit eosinophil survival-enhancing activity which can be blocked by antibodies to IL-5, GM-CSF, and IL-3. Although serum MBP elevations are frequently associated with peripheral eosinophilia and the release of eosinophil proteins into patient’s serum, urine, and tissues due to the release of IL-5, presumably from activated T lymphocytes (15, 16, 25). More recently, other cytokines such as IL-4 have been studied in preclinical and clinical settings as possible antitumor agents. IL-4 is an important growth factor critical to the activation of a subset of T-helper cells (Th2) involved in antibody production (IgG1, IgE) and eosinophil activation (26). A murine tumor model utilizing transfection of tumor cells with IL-4 cDNA has demonstrated eosinophil infiltration at the site of rejecting tumor and in vivo depletion of eosinophils abrogates the antitumor effect (7, 8).

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The mechanism by which IL-4 administration leads to systemic activation of eosinophils is likely a result of IL-4-
mediated activation of helper T cells of the Th2 subset (26). A clonal proliferation of Th2 CD4+ T cells has been characterized in the blood of a patient with hypereosinophilic syndrome (28). IL-4 alone has no direct effect on in vitro eosinophil degranulation or survival (29). Although we have no direct evidence for activation of these CD4+ T cells, they are known to require IL-4 for their in vitro activation and upon activation can secrete IL-5 and induce eosinophilia in animal models (9, 26). As previously reported, patients in this trial did not demonstrate activation of their peripheral CD3+ T cells based on the expression of CD45RO or CD25 (19). The CD4 subset of T cells was not separately evaluated by flow cytometry. GM-CSF and IL-3 are produced by both Th1 and Th2 cells (30). In this trial, sera from patients receiving IL-4 caused enhancement of eosinophil survival which could be partially neutralized by anti-IL-5, anti-GM-CSF, and possibly anti-IL-3. It is therefore likely that in vivo IL-4 results in Th2-derived secretion of eosinophil-active cytokines including IL-3, IL-5, and GM-CSF. The IL-4-induced cytokines are likely responsible for the increase in peripheral eosinophil counts, increase in serum and urine MBP, tissue deposition of MBP, and sera eosinophil survival-enhancing activity. In vitro and in animal systems, IL-4 has been shown to induce VCAM-1 expression on endothelial cells (31, 32). VCAM-1 expression appears to be critical to eosinophil binding to endothelium and trafficking into sites of inflammation (33). Although we have not examined whether IL-4 administration to humans can induce endothelial expression of VCAM-1, this is likely, based on the tissue infiltration by eosinophils and the release of MBP seen on the skin biopsies obtained from patients with rashes.

What is the role for eosinophil activation in the clinical effects of cytokines, especially IL-4? At toxic doses of IL-4, patients experienced a vascular leak syndrome similar to the syndrome associated with IL-2 of weight gain, peripheral edema, pleural effusions, oliguria, and a diffuse skin rash. The peripheral tissue infiltration of eosinophils and release of MBP and other cytotoxic proteins could damage endothelial integrity and lead to this syndrome (34). Since both peripheral eosinophilia and release of MBP have been observed in association with IL-4 and IL-2 therapy, eosinophil mediation of cytokine toxicity could be targeted as a means to ameliorate the severe side effects. On the other hand, the degree of eosinophilia did not clearly correlate with clinical toxicity in this small patient population. Finally, could IL-4 mediate tumor regression through eosinophil activation? Some murine experiments suggest that local IL-4 secreted at tumor sites requires eosinophil infiltration for tumor rejection (8). In other animal studies, IL-4 gene transfection of tumors have failed to induce eosinophils at the site of tumor rejection, and tumor rejection appeared to require IFN-γ produced locally (35, 36). Last, IL-5 transfection of tumor cells can induce a massive local infiltration of activated eosinophils without inducing protective immunity (37). IL-4 has not been shown to be an effective anticancer agent in humans up to this time (38, 39). Trials are ongoing to better define its clinical effectiveness in various tumor types. This study dem-
onstrated no objective (>50%) tumor regressions in the 17 patients enrolled (19). A single patient had a prolonged minor response. This patient with renal cell cancer was treated at the lowest dose of IL-4 (40 μg/m²), but did show some increase in eosinophils and release of MBP. Patients receiving IL-2 as an antitumor therapy develop eosinophilia (15, 16, 25). The clinical significance of the eosinophilia is not known. Results from this study suggest that a closer evaluation of other inflammatory cells, such as eosinophils, is warranted and could potentially yield clues to the mechanism of cytokine-induced tumor regression in humans.

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Evidence for eosinophil activation in cancer patients receiving recombinant interleukin-4: effects of interleukin-4 alone and following interleukin-2 administration.

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