In Vivo Biologic Effects of PIXY321, a Synthetic Hybrid Protein of Recombinant Human Granulocyte-Macrophage Colony-Stimulating Factor and Interleukin-3 in Cancer Patients With Normal Hematopoiesis: A Phase I Study


PIXY321 is a novel fusion protein of recombinant human granulocyte-macrophage colony-stimulating factor and interleukin-3 (IL-3) that exhibits biologic effects of both its parent cytokines in vitro and in preclinical studies. To evaluate the clinical safety and hematopoietic effects of this hybrid cytokine, PIXY321 was administered by subcutaneous injection twice daily at doses of 25 to 1,000 μg/m2/day over 14 days to 24 patients with sarcoma before chemotherapy as part of a phase I trial. The treatment was associated with significant increases in white blood cell, neutrophil, platelet, and reticulocyte counts (all P < .001). The increase in neutrophil count was dose-related and was seen during treatment with the cytokine, whereas the increase in platelet count was gradual and peaked after the cessation of the cytokine treatment and was not clearly dose related. PIXY321 treatment also increased bone marrow (BM) cellularity and the percentage of BM cells in S phase (P < .001). In addition, there was a significant increase in the number of CFU-GM cells and committed and multipotential progenitors in the peripheral blood. The ex vivo expansion capacity of peripheral blood and BM progenitor cells was preserved after the in vivo treatment with PIXY321. The treatment was well tolerated, with the most common side-effect being injection site reactions. The results of this study show the biologic and clinical activity of a genetically engineered fusion molecule of two hematopoietic cytokines in humans with normal hematopoietic function.

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Their complementary in vivo biologic effects and cross-competition for receptor binding prompted the development of a fusion molecule of GM-CSF and IL-3 in an attempt to create a therapeutic agent with multilineage activity. In vitro studies have shown that PIXY321 binds to cell lines that express specific receptors for GM-CSF or IL-3 with affinities similar to the native hematopoietins. However, on cell lines that express both GM-CSF and IL-3 receptors, PIXY321 exhibits enhanced binding capacity compared with IL-3. PIXY321 stimulates both committed and multipotential progenitor cells in vitro and mediates enhanced biologic effects when compared with GM-CSF and IL-3, alone or in combination. PIXY321 has also been shown to support various stages of megakaryocytogenesis in vitro. In preclinical studies, PIXY321 was found to accelerate granulocyte recovery similar to GM-CSF and platelet recovery similar to IL-3 in pri mates subjected to sublethal radiation. On the basis of these observations, we conducted a phase I-II clinical-laboratory investigation of PIXY321 in cancer patients at risk of chemotherapy-induced multilineage myelosuppression. This phase I/II study was divided into two parts, ie, prechemotherapy (phase I part) and postchemotherapy (phase II part). The effects of PIXY321 postchemotherapy have recently been reported. The objectives of the present (phase I) study were to assess the biologic effects and clinical tolerance of this novel fusion protein in patients with normal hematopoiesis when administered before chemotherapy.

Patients and Methods

Chemotherapy-naive patients with sarcoma who were suitable candidates for subsequent chemotherapy were eligible for this trial. Patients were required to have Karnofsky performance status of ≥60% and adequate renal function (serum creatinine level, ≤1.5 mg/dL), adequate hepatic function (total bilirubin level, ≤1.5 mg/dL), and a life expectancy of at least 3 months. Written informed consent was obtained from all patients before entry onto the study in accordance with institutional guidelines.

Recombinant Human PIXY321

PIXY321 is a fusion protein that consists of active domains of recombinant human GM-CSF and IL-3 connected by a flexible...
amino acid linker sequence. The recombinant PIXY321 used in this study was a glycoprotein produced in yeast (Saccharomyces cerevisiae) and provided by Immunex Corp (Seattle, WA). The purified protein has a molecular weight of approximately 35 kD and a specific activity of 1 × 10⁶ U/mg of protein using an erythroleukemia cell line (TFI) bioassay.

**Study Design**

During the phase I dose-escalation portion of the study, the subject of this report, PIXY321, was administered before chemotherapy by subcutaneous injection twice daily over 14 days. At least three patients each were entered at seven different dose levels, including 25, 50, 125, 250, 500, 750, and 1,000 ng/m2/day. The treatment was to be discontinued if any patient experienced intolerable side-effects or rapid progression of disease during treatment.

**Clinical and Laboratory Monitoring**

Before and during the course of the clinical trial, patients were monitored with an examination of their history, physical examinations, and laboratory tests, including a complete blood cell count including differential counts, serum chemistry, coagulation test, arterial blood gas analysis, chest radiograph, and an EKG. Blood counts and reticulocyte counts were obtained three times per week. Bone marrow (BM) aspiration and biopsy were performed before and at the end of the first course of PIXY321 and approximately 36 hours after discontinuation of PIXY321. BM samples were examined for cellularity, morphology, and myeloid-erythroid cell ratios and were assayed for cell cycle status (BrdU flow cytometry) and content of CD34⁺ cells (flow cytometry) as described below. In addition, blood samples were assayed for hematopoietic progenitor cell number, underwent progenitor cell expansion assays (delta assay), underwent CD34⁺ cell analysis (flow cytometry), and were assayed for neutrophil function (nitroblue tetrazolium [NBT] reduction).

**Hematopoietic Progenitor Cell Assay**

Assays for colony-forming unit granulocyte-macrophage (CFU-GM); burst-forming unit-erythroid (BFU-E); and colony-forming unit granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) using low-density BM and peripheral blood (PB) cells were performed as previously described. Briefly, CFU-GM were scored in 0.3% agar-culture media plates after 14 days of incubation in the presence of 100 U/mL recombinant human (rh) GM-CSF to detect more mature CFU-GM or 100 U/mL of rhGM-CSF + 50 ng/mL of rh steel factor (SLF; c-kit ligand) to detect more immature subsets of CFU-GM. BFU-E and CFU-GEMM colonies were scored in 0.9% methylcellulose culture media plates after 14 days of incubation in the presence of 1 U/mL of rhEPO + 100 U/mL of rhIL-3 to detect more mature BFU-E or 1 U/mL rhEPO + 100 U/mL rhIL-3 + 50 ng/mL rhSLF to detect more immature subsets of BFU-E and CFU-GEMM. Cultures were incubated at 37°C in a humidified atmosphere.

**Hematopoietic Progenitor Cell Expansion Assay (Delta Assay)**

CFU-GM and BFU-E expansion in liquid cultures were performed as described previously. Briefly, mononuclear PB and BM cells (1 × 10⁶) were cultured in 10 mL of McCoy's medium (GIBCO, Grand Island, NY) supplemented with 20% heat-inactivated fetal calf serum (Intergen, New York, NY), PIXY321 (100 ng/mL concentration), and SLF (1 µg/mL concentration). The cultures were incubated at 37°C in a humidified atmosphere. On day 4, the cultures were demixed and repopulated with nonadherent cells and an equal volume of fresh media with the appropriate growth factors was added. To calculate the input values, an aliquot of freshly isolated mononuclear cells was assayed for CFU-GM and BFU-E in methylcellulose cultures before liquid culture. Clonal growth was stimulated with 100 ng/mL of PIXY321 for CFU-GM and PIXY321 + 2 U/mL of EPO for BFU-E. Cells harvested from liquid cultures on day 8 were washed with phosphate-buffered saline (PBS), resuspended in media, and then plated in methylcellulose culture medium to calculate the output values. CFU-GM and BFU-E were counted after 14 days of incubation. Total CFU-GM and BFU-E content was calculated and compared with input numbers to determine the level of expansion.

**Flow Cytometric Analysis of CD34⁺ Cells**

**Antibodies.** Fluorescein-conjugated anti-CD34 was obtained from AMAC, Inc (Westbrook, ME). The matching conjugated isotype control antibody was obtained from Coulter Immunology (Hialeah, FL).

**Immunofluorescence.** Immunofluorescence was performed on 0.1 mL samples of blood that had been drawn into heparinized plastic syringes. Saturating amounts of anti-CD34 MoAb or the isotype controls were added directly to the blood in polypropylene tubes and allowed to react at 4°C for 30 minutes. The blood was washed with Dulbecco's PBS (GIBCO) and red blood cells were lysed using FACS lysis solution (Becton Dickinson). The leukocytes were then washed and fixed by 1% paraformaldehyde. All samples were analyzed by flow cytometry on the same day that the blood was drawn.

**Flow cytometry was performed on an Epics Profile I (Coulter Cytometry, Hialeah, FL) with an argon laser tuned to 488 nm and with three fluorescence channels. Leukocyte populations were selected for fluorescence analysis based on a combination of forward angle and side angle light scatter characteristics. Fluorescence gates for the determination of positive cells were set based on the isotype control samples. The mean intensity of fluorescence was converted to mean channel number based on a 256-channel histogram.**

**NBT Reduction Test**

To determine the effects of PIXY321 on neutrophil function, an NBT reduction test was performed. Blood samples were obtained from patients four times: before PIXY321 treatment (pre), on days 7 (mid) and 14 (end) of PIXY321 administration, and 7 days after the end of treatment (post). Each patient was paired with a single control donor (adult volunteer) and the patient/control pair was examined at each of the time points, as described above. Heparinized blood was diluted 1:1 with PBS and separated by Ficoll-Hypaque centrifugation dextran sedimentation-hypotonic lysis. The polymorphonuclear leukocyte fraction was then adjusted to 5 to 10 × 10⁶/mL in Hanks' Balanced Salt Solution and mixed 1:1 with Hanks' Balanced Salt Solution containing 0.2% NBT and 40 ng/mL phorbol myristate acetate. After 15 minutes of incubation at 37°C, slides were prepared with a Cytospin (Shandon Southern, Sewickley, PA), air dried, and counterstained with 0.25% safranin. Slides were then examined microscopically under oil, and the percentage of polymorphonuclear leukocytes stained with blue-purple formazan was estimated from a 100-cell count. Cytospin preparations of fresh cells were also made, air dried, stained with modified Wright Giemsa (Diff-Quick Scientific Products, McGaw Park, IL), and used for differential WBC counts.

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RESULTS

Twenty-five patients were entered onto the trial. One patient did not receive the treatment outlined in the protocol because his diagnosis was found to be lymphoma, making him ineligible for the study. Twenty-four patients (14 men and 10 women) with the diagnosis of sarcoma were treated on this trial, and all were considered evaluable for clinical tolerance and response to PIXY321. The median age of this patient group was 45 years (range, 19 to 74 years), and the Karnofsky performance status was 95 (range, 90 to 100). Seven patients had received prior radiation therapy and 17 had had prior surgery.

Hematopoietic Response

WBCs. Treatment with PIXY321 was associated with significant increases (Table 1) in WBC count (baseline mean, 6.6 × 10^3/μL; maximal mean count during treatment, 15.5 × 10^3/μL; P < .001). The increase in WBC was primarily due to mature neutrophilic granulocytes (ANCs; baseline mean, 4.4 × 10^3/μL; maximal mean, 11.8 × 10^3/μL; P < .001). The increase in neutrophil count was dose-related (Fig 1). The increase in WBCs and ANC was biphasic, ie, it increased within 48 hours after the treatment was started (Fig 1). This initial increase was followed by a brief decline and then a second peak. Although significant increases in band forms of neutrophils were seen (Table 1), more immature forms such as promyelocytes, myelocytes, or metamyelocytes were rarely observed in the PB. Whereas modest increases in other myeloid cells (ie, monocytes, eosinophils, and basophils) and lymphoid cells were also seen (Table 1), the increase in neutrophils accounted for most of the WBC response. All patients exhibited an increase (1.3- to 5.7-fold) in WBC values during the 14-day PIXY321 administration in a dose-related manner (P < .001). On the last day of treatment (day 14), the mean WBC values for all patients at each dose level ranged from 7,000 to 9,000/μL at less than 250 μg/m2/d, 12,000 to 13,000/μL at 250 to 500 μg/m2/d, and 17,000 to 19,000/μL at 750 to 1,000 μg/m2/d. The counts gradually returned to a pretreatment level within 1 week of discontinuation of the cytokine treatment.

Platelets. PIXY321 treatment also resulted in significant increases (1.2- to 4.3-fold) in platelet counts (baseline mean, 312 × 10^3/μL; maximal mean, 430 × 10^3/μL; P < .001). The increase in platelet count was seen at all dose levels (Fig 2) in 19 of 24 patients and did not appear to be clearly dose-related (P = .16). The kinetics of the platelet response were different from those of the WBC and neutrophil response. The increase in platelet count was gradual and generally peaked 1 week (median day 20) after the discontinuation of PIXY321 treatment (Fig 2). The mean platelet counts remained higher on day 21 than at baseline for all dose levels. The mean platelet values for all patients at each dose level ranged from 256,000 to 363,000/μL at baseline, from 256,300 to 442,300/μL on the last day of the treatment (day 14), and from 311,500 to 476,800/μL 1 week after the discontinuation of the cytokine (day 21).

Reticulocytes. The reticulocyte count also increased (baseline mean absolute corrected reticulocyte count, 6.8 × 10^9/μL; maximal mean, 14.9 × 10^9/μL; P < .001) during

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Table 1. Effects of PIXY321 on WBC Counts and Differentials

<table>
<thead>
<tr>
<th>WBC</th>
<th>Pre</th>
<th>Post</th>
<th>Mean</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>7.0</td>
<td>7.8</td>
<td>4.3</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>Basophils</td>
<td>0.3</td>
<td>1.7</td>
<td>0.6</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>2.5</td>
<td>2.7</td>
<td>2.5</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0.7</td>
<td>0.8</td>
<td>0.8</td>
<td>&lt; .001</td>
</tr>
</tbody>
</table>

Mean ± SEM values are shown. Values are PB counts × 10^9/μL.

Abbreviation: Post, the maximal response in counts during first cycle of PIXY321 treatment.
PIXY321 treatment and peaked at a median of day 11. Despite the frequent phlebotomies performed during the study, hematocrit values remained stable during treatment.

**Neutrophil Functions**

**NBT reduction test.** At baseline, the percentages of NBT-positive neutrophils observed in patients (97% ± 1%) and controls (93% ± 2%) were the same (P = .152). The percentages of NBT-positive neutrophils did not change in either the controls (94% ± 2% [day 7] and 96% ± 1% [day 14]) or the patients (96% ± 1% [day 7] and 97% ± 1% [day 14]) during treatment with PIXY321. Studies that examined locomotive, respiratory burst, and phagocytic responses of polymorphonuclear leukocytes during PIXY321 administration have been previously reported.14

**Effects on Mobilization of Progenitor Cells**

**Hematopoietic progenitor cell assay.** To determine whether in vivo treatment with PIXY321 expands the circu-
lating pool of hematopoietic progenitor cells, both mature (ie, CFU-GM and BFU-E detected by growth in vitro in response to EPO + IL-3) and more immature subsets (CFU-GM, BFU-E, and CFU-GEMM detected by growth in vitro in response to SLF + EPO + IL-3) of progenitor cells were assessed before, during, and after PIXY321 administration. There was a significant increase in the number of both mature and immature subsets of committed (CFU-GM and BFU-E) and multipotent (CFU-GEMM) progenitor cells in PB during PIXY321 administration (Table 2). The magnitude of the response was heterogenous in different patients treated at the same doses (Table 3). The maximal increases were seen up to ninefold for CFU-GM, 15-fold for BFU-E, and 12-fold for CFU-GEMM using IL-3, EPO, and SLF as the growth stimuli in vitro. The time of maximum response also varied; however, the peak effect was generally seen around day 7 to 9 and was followed by either a slight decline or plateau effect (Table 2) such that the progenitor cell number on day 21 (1 week after the discontinuation of cytokine) remained higher than the baseline value. There appeared to be some dose response (Table 3) because the peak increase in mature progenitor cells was greater at higher doses (500 to 1,000 μg/m²/d) than at lower doses (25 to 250 μg/m²/d).

Flow cytometric analysis of CD34+ cells in PB. To determine whether treatment with PIXY321 results in the release of CD34+ cells, PB was examined for the number of CD34+ cells. The number of circulating CD34+ cells in PB significantly increased over baseline (0.8- to 9.4-fold) by days 7 through 10 and remained elevated during 14 days of treatment with PIXY321 (Table 2). On day 21 (ie, 1 week after the completion of PIXY321), the CD34+ cell concentration was still significantly higher than the baseline value (P < .01). The maximum increase in circulating CD34+ cells also appeared to be dose-related (Table 3).

Table 2. Peripheral Blood Progenitor Cells and CD34+ Cells During PIXY321 Administration

<table>
<thead>
<tr>
<th>Study Day</th>
<th>Baseline</th>
<th>2-6</th>
<th>7-9</th>
<th>10-13</th>
<th>14-15</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of Cells</td>
<td>(n)</td>
<td>(n)</td>
<td>(n)</td>
<td>(n)</td>
<td>(n)</td>
<td>(n)</td>
</tr>
<tr>
<td>Mature* progenitors (EPO + IL-3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFU-GM</td>
<td>51 ± 13</td>
<td>71 ± 19</td>
<td>132 ± 27†</td>
<td>76 ± 14</td>
<td>99 ± 33</td>
<td>71 ± 29</td>
</tr>
<tr>
<td>BFU-E</td>
<td>120 ± 23</td>
<td>162 ± 43</td>
<td>248 ± 341</td>
<td>214 ± 566</td>
<td>187 ± 40</td>
<td>281 ± 118†</td>
</tr>
<tr>
<td>Immature* progenitors (SLF + EPO + IL-3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFU-GM</td>
<td>89 ± 17</td>
<td>116 ± 29</td>
<td>173 ± 25†</td>
<td>90 ± 14</td>
<td>117 ± 29</td>
<td>157 ± 51</td>
</tr>
<tr>
<td>BFU-E</td>
<td>95 ± 21</td>
<td>135 ± 35</td>
<td>243 ± 47†</td>
<td>156 ± 39</td>
<td>153 ± 27</td>
<td>174 ± 63</td>
</tr>
<tr>
<td>CFU-GEMM</td>
<td>66 ± 13</td>
<td>86 ± 16</td>
<td>106 ± 25</td>
<td>77 ± 20</td>
<td>88 ± 24</td>
<td>119 ± 44</td>
</tr>
<tr>
<td>CD34+ cells†</td>
<td>106 ± 24</td>
<td>161 ± 40</td>
<td>212 ± 29†</td>
<td>283 ± 37</td>
<td>248 ± 33†</td>
<td>203 ± 19†</td>
</tr>
</tbody>
</table>

Data represent the mean of all patients examined at all doses at each time point.
* Mean ± SEM values for number of colonies per milliliter of blood.
† Mean ± SEM values for absolute number of CD34+ cells per microliter of blood.
‡ P < .01.
§ P < .07.
| P < .001.

Table 3. Mobilization of Progenitor Cells Associated With PIXY321 Treatment: Peak Response

<table>
<thead>
<tr>
<th>Dose of PIXY321 (μg/m²/d)</th>
<th>CFU-GM</th>
<th>BFU-E</th>
<th>CFU-GM</th>
<th>BFU-E</th>
<th>CFU-GEMM</th>
<th>CD34+ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low dose (25-250)</td>
<td>(n = 8)</td>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td>(n = 4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.9 (0.6-20)</td>
<td>1.9 (1.1-16.5)</td>
<td>2.7</td>
<td>3.8</td>
<td>3.3</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>1.2 (1.6-22)</td>
<td>1.2 (1.2-12.5)</td>
<td>6.2</td>
<td>4.4</td>
<td>3.1</td>
<td>4.0</td>
<td>3.3</td>
</tr>
<tr>
<td>High dose (500-1,000)</td>
<td>(n = 11)</td>
<td>(n = 11)</td>
<td>(n = 11)</td>
<td>(n = 6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.9 (1.1-9.4)</td>
<td>1.1 (1.28)</td>
<td>6.2</td>
<td>4.4</td>
<td>3.1</td>
<td>4.0</td>
<td>3.3</td>
</tr>
<tr>
<td>1.2 (1.3-8.1)</td>
<td>0.9-15.2</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1.1 (1.3-11.8)</td>
<td>3.2-9.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Data shown represent median (range) values for maximum response at lower and higher doses.
† In vitro stimulus IL-3 + EPO.
‡ In vitro stimulus SLF + IL-3 + EPO.
**Effects on BM**

To better understand the cellular basis for the hematopoietic responses in the PB, we examined BM cellularity, myeloid-erythroid (M:E) ratio, frequency of CD34+ cells and progenitor cells, and the proliferative rate of BM cells before and during in vivo treatment with PIXY321.

**BM morphology.** To determine whether the increase in blood count was associated with stimulation of hematopoiesis in the BM, aspiration and biopsy were performed before, during, and after PIXY321 administration in 23 patients. The treatment was associated with an overall modest increase in BM cellularity (from mean 35% to 45%; P < .001, n = 23). The increase in cellularity appeared to be dose-related (Table 4) and was greater 2 days after the discontinuation of the cytokine. Six of twelve patients treated at higher doses showed a increase in cellularity of 40% to 129% over the baseline values. Treatment also resulted in a shift to the left of myelopoiesis with a significant increase in promyelocytes, myelocytes, and eosinophils. There was no significant change in the M:E cell ratio. The number of megakaryocytes/high power field increased significantly in a dose-related manner (the specific effects of PIXY321 on megakaryocytepoiesis will be the subject of a separate report).

**BM CD34+ cells and progenitor cells.** BM cells were analyzed for CD34+ cells both before and after treatment with PIXY321 in 21 patients. There was no significant change in the percentage of CD34+ cells (baseline, 4.3% ± 0.7%; posttreatment, 3.2% ± 0.4%; P = .16) in the BM.

**Proliferation of BM cells.** To determine whether the increase in blood counts was associated with changes in the proliferative status of the BM cells, BrdU flow cytometry was performed on paired samples, 15 before and 15 after the PIXY321 treatment. The percentage of BM cells in S-phase, as measured by BrdU flow cytometry, was significantly increased after treatment with PIXY321 (from 6% to 9%; P < .001, n = 15) at all doses (Table 4). The increase in the percentage of cells in S phase was consistently observed in each patient after PIXY321 treatment. The frequency of cycling progenitor cells (both committed [CFU-GM and BFU-E] and multipotential [CFU-GEMM] progenitor cells), as measured using the high specific activity tritiated thymidine suicide technique, was also increased markedly after treatment with PIXY321.11

**Expansion of Progenitor Cells (Delta Assay)**

To determine whether in vivo treatment with PIXY321 would affect the capacity of PB and BM progenitor cells to expand ex vivo in response to a combination of hematopoietic cytokines (PIXY321 + SLF), PB and BM samples were analyzed for myeloid progenitor cells (CFU-GM) by the delta assay both before and during (day 14) PIXY321. As shown in Table 5, although there was a trend for a higher expansion value for both PB and BM progenitor cells after treatment with PIXY321 at lower doses (25 to 250 μg/m2) than at baseline, these changes were not statistically significant.

**Antibody Analysis**

To determine whether in vivo treatment with PIXY321 was associated with the development of antibodies that might interfere with its biologic effect, serum samples from 22 patients were screened for the development of anti-PIXY321 antibodies by enzyme-linked immunosorbent assay (ELISA). A total of 8 of the 22 patients (0 of 6 at 25 to 50 μg/m2, 2 of 6 at 125 to 250 μg/m2, and 6 of 10 at 500 to 1,000 μg/m2 dose level) tested positive using ELISA for PIXY321 antibodies. Of these 8 patients, three were positive using ELISA for both GM-CSF and IL-3, three were negative for both, and two were positive only for IL-3. All 8 samples were negative when tested against *Escherichia coli*-derived (nonglycosylated) GM-CSF using Western blot analysis, suggesting that the antibodies are directed to O-linked glycosylation of the PIXY321 molecule. However, all samples were negative for neutralizing antibody when tested in a bioassay (BM colony-forming assay), suggesting that the antibody response would not impair in vivo biologic activities.

**Clinical Tolerance to PIXY321**

Treatment with PIXY321 was generally well tolerated. The most common side-effect observed during PIXY321
administration was a local skin reaction at the injection site that was characterized by erythema, mild induration, warmth, and occasional itching. In addition, about half of the patients experienced constitutional symptoms such as bone pain, myalgia, headache, malaise, and low-grade fever. These symptoms were generally mild to moderate in intensity and transient in nature. The fever generally lasted for 1 to 2 days during drug administration. No dose-limiting toxicity was encountered with doses up to 1,000 μg/m²/d.

**DISCUSSION**

PIXY321 was constructed to combine the complementary biologic effects of two cytokines (GM-CSF and IL-3) into a single therapeutic agent with multilineage activity. There are several questions to be addressed in studying this molecule in humans. First, would this hybrid cytokine retain biologic effects of both its component cytokines in vivo? Second, would administration of this synthetic protein result in an unusual toxicity profile or immunogenicity? The purpose of this phase I study was to evaluate the in vivo biologic effects of PIXY321 (GM-CSF/IL-3 fusion protein), including clinical tolerability and immunogenicity, and to define the nature of the hematopoietic response to this synthetic hybrid protein in patients with normal baseline hematopoiesis before chemotherapy.

In this group of patients, the response to PIXY321 was characterized by significant increases in WBC counts, platelet counts, and reticulocytes. The magnitude, spectrum, and the kinetics of hematopoietic response suggest that PIXY321 exhibited hybrid biologic effects of both its component cytokines, GM-CSF and IL-3, in vivo. Similar to GM-CSF, the increase in WBC count was dose-related and was seen during the period of cytokine administration. However, the magnitude of the WBC response was more modest than that produced by GM-CSF. The increases in WBC were predominated by an increase in mature neutrophils, although modest increases in other myeloid (ie, eosinophils, monocytes, and basophils) as well as lymphoid cells were also seen. The kinetics of the increases in WBCs and ANC were biphasic. The initial peak was observed during the first 2 to 3 days of drug administration and was probably due to demargination of cells or mobilization of cells from the BM. The rapid increase in leukocytes has been previously seen with the late-acting myeloid growth factors such as G-CSF and GM-CSF but not with earlyacting growth factors such as IL-3. The second peak was seen during the second week of drug administration and was most likely related to the proliferative effect on the BM. This latter finding is further supported by several observations. First, the response in PB count was associated with increases in BM cellularity. Second, there was both in vivo and ex vivo evidence for induced proliferation of BM precursor cells (using flow cytometric analysis) as well as progenitor cells (using colony-forming assays). The WBC gradually returned to the baseline level more than 1 week after discontinuation of the cytokine.

Similar to IL-3, significant increases in platelet counts were seen after PIXY321 treatment. The kinetics of response were different for leukocytes and platelets. The increase in platelet count was gradual, modest, and peaked 1 week after cessation of treatment with PIXY321. This observation was similar to that obtained with IL-3. Whereas there was no clear dose response in platelets before chemotherapy in these patients with normal baseline hematopoiesis, after chemotherapy, reduction in cumulative thrombocytopenia by PIXY321 was dose-related, with the optimal effects seen at 750 μg/m²/d. The slower kinetics of the platelet response compared with the neutrophil response along with the increased number of megakaryocytes in the BM indicate that the increase in platelet count may have been principally related to the proliferative rather than maturation effect of this cytokine on megakaryocytopoiesis.

Both GM-CSF and IL-3 exhibit burst-promoting activity and stimulate erythropoiesis in vitro. Whereas PIXY321 treatment was associated with an increase in reticulocyte numbers, this did not translate into an increase in hematocrit during the prechemotherapy phase. Two possible mechanisms may explain this finding. First, there is a time lag for development of mature red blood cells from responsive progenitor cells. Thus, an increase in the hematocrit level might have been difficult to observe because these patients received only one cycle of PIXY321 before chemotherapy. Secondly, these patients had normal hematocrit levels before receiving PIXY321. Interestingly, improvement in the hematocrit levels along with reductions in red blood cell transfusion requirement were observed during multiple cycles of chemotherapy with PIXY321 support. Furthermore, in our separate clinical trial of PIXY321 in patients with BM failure conditions related to intrinsic disease processes, durable responses in erythropoiesis and thrombopoiesis have been seen in a number of patients. These findings suggest that the nature and magnitude of hematopoietic responses to the hematopoietic cytokines might be different in normal versus suppressed hematopoiesis.

One of the most interesting and clinically relevant biologic properties observed with the hematopoietic growth factors (G-CSF and GM-CSF) is their ability to mobilize hematopoietic progenitor cells in vivo. This has facilitated the harvest of a large number of circulating progenitor cells for clinical application with dose-intensive chemotherapy. In the present study, administration of PIXY321 resulted in a significant increase in the circulating pool of both committed and multipotential progenitor cells. Although there was some individual variation in the time for peak response, progenitor cell numbers were consistently increased on days 7 through 10 for most patients. The timing of response in progenitors cells was also mirrored in the increase in the number of CD34+ cells seen in the PB. However, the magnitude of mobilization appeared more modest in this study as compared with that observed with GM-CSF or G-CSF. Whether there are clinically relevant differences in the quality of progenitor cells (ie, early v late progenitors) mobilized by PIXY321 compared with lineage-specific molecules remains to be evaluated in clinical trials. Optimizing the dose and schedule of PIXY321 administration to maximize mobilization is a focus of ongoing studies.

Although cytokines have enhanced our ability to obtain target cells (progenitors) responsive to hematopoietic growth factor, the host response is not always optimal to collect a sufficient number of progenitor cells for transplantation purposes. Ex vivo expansion of the progenitor cells could
provide a more readily available cell source under those circumstances. For this reason, it was of interest to determine whether in vivo treatment with PIXY321 would affect the ability to expand progenitor cells ex vivo, perhaps by differentiating the available responsive progenitor cells. Our finding showed that, despite in vivo treatment with PIXY321, the progenitor cells could be expanded ex vivo and suggests that short-term administration of PIXY321 in vivo has no deleterious effect on the more primitive hematopoietic cells that are responsible for ex vivo expansion.

Treatment with PIXY321 when administered by the subcutaneous route up to 1,000 μg/m²/d in two divided doses was generally well tolerated. The nature of the side-effects was similar to that observed with GM-CSF (constitutional symptoms) and IL-3 (local skin reactions and constitutional symptoms). The side-effects of PIXY321 were generally mild to moderate in severity and no dose-limiting toxicity was encountered with doses up to 1,000 μg/m²/d. This finding is of interest in light of the observation that the maximum tolerated dose for IL-3 is defined as 10 to 15 μg/kg/d.

One possible concern with the use of genetically engineered hybrid molecule relates to its potential immunogenicity. Whereas 36% of the patients were found to test positive for antibodies on an ELISA, all were negative for the development of neutralizing antibodies. In addition, all samples were negative against E coli-derived GM-CSF, suggesting that these antibodies were most likely directed towards carbohydrate moieties of the molecule. Furthermore, in the initial clinical trials, patients have shown hematopoietic benefit during multiple cycles.

In summary, the results of this study show that PIXY321, a hybrid protein of GM-CSF and IL-3, elicits biologic effects characteristic of both of the parent cytokines. The magnitude of response was modest. However, the nature of hematopoietic response was multilineage, consistent with the effects of GM-CSF and IL-3 on hematopoietic pathways. This finding suggests that PIXY321 might be useful to increase the reservoir of early hematopoietic cells that can be called on after chemotherapy. Because the production of mature blood cells requires the hierarchical interplay of cytokines, combinations of early and late-acting growth factors might be required to achieve maximal stimulation of progenitor cells. PIXY321 may provide a stimulus for early to intermediate stages of hematopoiesis that could be further enhanced by lineage-specific hematopoietins such as G-CSF, erythropoietin, or thrombopoietin when biologic effects in specific lineages are more desired. The potential therapeutic role for this novel cytokine is currently being investigated in the setting of prevention and treatment of hematopoietic suppression.

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In vivo biologic effects of PIXY321, a synthetic hybrid protein of recombinant human granulocyte-macrophage colony-stimulating factor and interleukin-3 in cancer patients with normal hematopoiesis: a phase I study

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