Efficacy of an Insulin-Like Growth Factor-Interleukin-3 Fusion Protein in Reversing the Hematopoietic Toxicity Associated with Azidothymidine in Mice¹

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ABSTRACT
The effect of 406, a novel fusion protein between the N-terminal sequence of the insect insulin-like peptide, bombyxin, human insulin-like growth factor II and mouse interleukin 3 was investigated in its capacity to abrogate the toxic effects of azidothymidine (AZT) in C57BL/6 mice. Mice receiving 2.5 mg/ml AZT in their drinking water were concurrently treated with daily s.c. injections of 14, 140 or 1400 ng 406 for 4 wk. AZT-treated mice had a lower total weight, hemoglobin content and white blood cells than non treated controls. 406 significantly increased the number of circulating white blood cells at all doses, and the optimal effects were observed at a dose of 140 ng/mouse. Using this optimal dose, 406 completely abrogated the AZT-mediated weight loss. The effects on erythroid cells depended on the severity of the AZT-induced anemia. The amounts of hemoglobin were equal or slightly lower than those of controls under conditions of mild anemia, but were significantly higher than controls under conditions of severe anemia. 406 significantly increased the number of all hematopoietic colony-forming cells in bone marrow and spleen, but the effects were particularly striking in granulocyte-macrophage precursors. Blood glucose levels did not change at optimal or suboptimal 406 doses but increased at a dose of 1.4 μg/mouse. These experiments demonstrate the usefulness of these IGF-cytokine fusion proteins, whose low cost production represents a significant advantage for future in vivo studies.

Zidovudine (AZT), a potent inhibitor of retroviral replication, was the first drug approved for the treatment of AIDS, and is currently used in AIDS patients despite its adverse hematologic effects (Mitsuya et al., 1985; Fischl et al., 1987; Richman et al., 1987). Several growth factors or cytokines have been evaluated in their capacity to counteract the AZT-mediated hematopoietic cytotoxicity. In vitro administration of IGF I in AZT-treated mice resulted in an increased hematocrit and an augmentation of the number of hematopoietic progenitor cells in spleen and bone marrow (Tzarfaty et al., 1994). IL-3, a cytokine with multilineage hematopoietic stimulatory activity, represents, in theory, an ideal therapeutic agent against a generalized decrease of all hematopoietic cells. HIV-1-infected patients with cytopenia responded to IL-3 in a phase I trial with increases in white blood cells, neutrophils and eosinophils whereas hemoglobin and plate-cell counts remained generally unaffected (Scadden et al., 1995). In vitro studies have clearly demonstrated the effectiveness of IL-3 in reversing AZT-mediated toxicity of all hematopoietic stem cells including erythroid precursors (Gallicchio and Hughes, 1993; Gogu et al., 1995). Unfortunately, these results cannot be fully reproduced in vivo and suggest that a complete reversal of AZT-mediated myeloid toxicity may require the additional use of other myelopoietic cytokines (Gallicchio et al., 1993).

We have previously found that BOMIGF, a chimera between the N-terminal sequence of the insect insulin-like peptide, bombyxin and the amino acids 9–67 of IGF II, is properly folded and secreted in insect cells when inserted in a recombinant baculovirus driven by the polyhedrin promoter (Congote and Li, 1994). Because other methods for large scale production of IGFs require refolding of the synthetic peptide, the baculoviral technique using BOMIGF represents a definitive advantage for the synthesis of properly folded, secreted chimeras between IGFs and other growth factors and cytokines. Cytokine chimeras such as PIXY321, a fusion protein of GM-CSF and IL-3, have been shown to be effective as therapeutic agents sharing the properties of both GM-CSF and IL-3, whereas hemoglobin and plate-cell counts remained generally unaffected (Scadden et al., 1995). In vitro studies have clearly demonstrated the effectiveness of IL-3 in reversing AZT-mediated toxicity of all hematopoietic stem cells including erythroid precursors (Gallicchio and Hughes, 1993; Gogu et al., 1995). Unfortunately, these results cannot be fully reproduced in vivo and suggest that a complete reversal of AZT-mediated myeloid toxicity may require the additional use of other myelopoietic cytokines (Gallicchio et al., 1993).

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and IL-3 (Vadhan-Raj et al., 1995). IGFs are often classified as progression factors complementing the mitogenic effects of many other (competent) growth factors. Therefore, they represent the ideal partner for the production of dimeric growth factors targeted primarily toward those cells containing receptors for both components of the chimera. We have recently obtained a chimera between BOMIGF and IL-3 with improved hematopoietic activity in vitro (DiFalco and Congote, 1997). The chimera had a higher mitogenic activity than IL-3 in cell cultures of the human hematopoietic cell line TF-1 and increased the number of macroscopic hematopoietic colonies in cultures of human peripheral blood in comparison with IL-3 or mixtures of IL-3 and BOMIGF. The objective of the present study is to assess the capacity of this hybrid molecule to abrogate the cytotoxic effects of AZT in vivo.

**Materials and Methods**

**Animals.** Male C57BL/6 mice (7 wk and older) were obtained from Charles River Canada, St. Constant, Quebec, Canada and had a weight of 18 to 22 g at the beginning of the experiments. AZT treatment (Zidovudine, GlaxoWellcome, Montreal, Canada) was initiated at a dose of 2.5 mg/ml in sterile drinking water. Controls received sterile water only. Daily water consumption was similar at the beginning of the experiments (6–8 ml per mouse) but decreased in AZT-treated mice to 3.9 ml per mouse at the end of the experiments. Mouse food was available ad libitum. All animal experiments were approved by the McGill University Animal Centre Committee.

**406 treatment.** 406, a chimera between BOMIGF and mouse interleukin 3 was isolated by a three-step HPLC procedure from culture supernatants of Sf9 cells infected with the baculovirus containing the cDNA of the chimera driven by the polyhedrin promoter (DiFalco and Congote, 1997). Aliquots of the preparations were lyophilized in siliconized tubes and kept at -20°C until use. Just before use, 406 was dissolved in 2 mM HCl. Dilutions were prepared in the same medium, neutralized with a 2% phosphate-buffered saline solution and immediately injected s.c. to prevent peptide adsorption in tubes or syringes. A total of 150 μl solution was injected per mouse. Control mice and AZT-treated mice received the same amounts of solution without 406. Injections were done daily 6 days per week. Because the experiments described in this work were done over a period of 8 mo and AZT-mediated anemia can vary considerably from one group to another, care was always taken that a matched group of control, AZT-treated and 406-treated mice were simultaneously housed, analyzed and used for the preparation of bone marrow or spleen cultures to allow paired comparisons with minimal variations.

**Blood sample analysis.** At the end of the treatment blood samples were taken by heart puncture in heparinized syringes. Hemoglobin was measured photometrically by the classical ferrocyanide method and total white cell count was done after lysis in 3% (v/v) acetic acid, 0.2% (w/v) methylene blue. In some experiments red cells were counted in a Coulter Counter Model ZB (Hialeah, FL) and hematocrit was measured using a Readacrit centrifuge (Clay Adams, Parsippany, NJ), calibrated with standards previously analyzed in a Tech- nicon H3 System (Technicon Instruments, Tarrytown, NY). Aliquots of the samples were centrifuged and the plasma frozen at -40°C for further determination of glucose concentrations (Trinder, 1969) using a commercial kit (Sigma, Oakville, Ontario, Canada).

**Quantitation of colony forming cells in spleen and bone marrow.** Bone marrow cell suspensions were obtained by flushing femurs and tibia with heparinized alpha medium (Gibco-Life Technologies, Burlington, Canada) supplemented with 2% (v/v) fetal bovine serum. Spleen cell suspensions were prepared after flushing the cells through 22-g needles. Red cells were eliminated by the ammonium chloride method (StemCell Technologies, Vancouver, Canada).

The cells were plated in methyl cellulose medium (Methocult M3434, Stem Cell Technologies) using 1.5 x 104 cells/ml of bone marrow cells per dish or 1.5 to 4.5 x 105 spleen cells per dish. The medium contained 0.9% (w/v) methycellulose, 15% (v/v) fetal bovine serum, 100 mM 2-mercaptoethanol, 2 mM L-glutamine, 1% (w/v) bovine serum albumin, 10 μg/ml bovine insulin, 200 μg/ml human transferrin, 3 U/ml erythropoietin, 10 ng/ml recombinant mouse IL-3, 10 ng/ml recombinant human IL-6 and 50 ng/ml recombinant mouse stem cell factor. Colonies were counted after 10 and 14 days of incubation (Tzarfaty et al., 1994; Eaves and Eaves, 1992). Colonies with diameters higher than 1 mm were considered as macroscopic and were counted directly without a microscope.

**Results**

Figure 1 shows the weight increases monitored weekly during AZT treatment in control mice (open squares), AZT-treated mice (open circles) and AZT-treated mice receiving at the same time daily injections of 140 ng 406 (closed squares). It can be seen that 406 completely abrogated the AZT-mediated weight lost. A significant weight gain was observed already after 2 wk of treatment.

Figure 2 shows the effects of 406 in AZT-treated mice on red cell count and hematocrit of peripheral blood. It is evident from these results that the action of 406 is particularly striking at the level of white blood cells. AZT treatment decreased the number of white blood cells and hemoglobin. 406 increased the white blood cell count but the amount of hemoglobin was apparently the same as in AZT-treated mice.

The experiments described above were done with a dose of 406 usually used in vivo with IL-3. To compare simultaneously the role of different doses of 406 on hemoglobin, white cell counts and total weight increases, we expressed the results as ratios of values observed in 406-treated mice over those of the AZT-treated controls of the same experimental group (fig 3). The values have been converted to a logarithmic scale to facilitate further the comparison of the three parameters tested. 14 ng/mouse 406 was able to increase the weight and the number of white cells, but only the effect on white cell number was statistically significant. All other results were significant, with the exception of the apparent decrease of hemoglobin concentrations. The optimal response was at the 100 ng range (140 ng/mouse). The highest dose tested (1400 ng/mouse)
The role of 406 in the number of hematopoietic precursors (CFU-GMs) and in cells producing macroscopic colonies.

Because there was an increase of erythroid cell progenitors in all experiments in bone marrow and spleen and 406 visibly increased the amounts on hemoglobin in many experiments, we investigated the reasons behind the variability of the erythropoietic response after 406 treatment. We found that there was a large variation in the degree of AZT-mediated anemia and that the highest increases in hemoglobin content were observed in those experiments in which AZT was caused...
Comparison of the effects of 406 in mice with different degree of anemia. The degree of anemia caused by AZT was measured in individual groups of mice by calculating the ratios on the hemoglobin concentrations of the AZT-treated mice as compared with those observed in the matched control mice. Experiments in which the amount of hemoglobin after AZT treatment was lower than 80% of the controls were classified in the group of "high" anemia. The effects of 406 (140 ng/mice) on white blood cell counts and hemoglobin concentrations was then expressed in each of these groups as the ratio of the amounts observed in 406-treated mice over the same amounts in mice treated with AZT alone. There was no statistical significant difference between both groups on the increase of white blood cells. The difference between the changes in hemoglobin concentrations caused by 406 in mice with high and low anemia was statistically significant (\(p < 0.04\), Mann-Whitney Test).

**Fig. 6.** Comparison of the effects of 406 in mice with different degree of anemia. The degree of anemia caused by AZT was measured in individual groups of mice by calculating the ratios on the hemoglobin concentrations of the AZT-treated mice as compared with those observed in the matched control mice. Experiments in which the amount of hemoglobin after AZT treatment was lower than 80% of the controls were classified in the group of "high" anemia. The effects of 406 (140 ng/mice) on white blood cell counts and hemoglobin concentrations was then expressed in each of these groups as the ratio of the amounts observed in 406-treated mice over the same amounts in mice treated with AZT alone. There was no statistical significant difference between both groups on the increase of white blood cells. The difference between the changes in hemoglobin concentrations caused by 406 in mice with high and low anemia was statistically significant (\(p < 0.04\), Mann-Whitney Test).

**Fig. 5.** In vitro formation of cell colonies from hematopoietic precursors of spleen cells collected 4 wk after treatment with 406 and AZT. Spleen cells were prepared from control mice (open bars), mice receiving AZT alone (hatched bars) or AZT plus 406 (140 ng/mouse, black bars) after 4 wk of treatment and plated in 3.5-mm cell culture dishes (150,000–500,000 cells/dish). The medium was semisolid methylcellulose containing all the growth factors required for optimal growth, as indicated in “Methods.” BFU-E, erythroid precursors. CFU-GM, granulocytic, macrophage precursors. Macros, macroscopic colonies. Mean ± S.E. from 16 experiments. The following differences were significant: BFU-Es, control cells vs. AZT-treated cells (\(p < 0.05\)) or 406-treated cells vs. AZT-treated cells (\(p < 0.01\)); CFU-GMs, control cells vs. AZT-treated cells (\(p < 0.05\)) or 406-treated cells vs. AZT-treated cells (\(p < 0.001\)). Analysis of variance, Student-Newman-Keuls test.

**Discussion**

IGFs and IL-3 stimulate practically all hematopoietic cell precursors in vitro (Shimon and Shpilberg, 1995; Clark and Kamen, 1987) and are therefore good candidates as therapeutic agents to counteract the hematopoietic toxicity caused by AZT. We have prepared a chimera of the N-terminal sequence of the insect insulin-like peptide, bombyxin, human insulin-like growth factor II and interleukin 3 (406) using the baculovirus expression system (DiFalco and Congote, 1997). The major advantage of this construct for in vivo studies is the possibility of obtaining, at low cost, large amounts of recombinant protein containing properly folded IGFs and which share the properties of a multilineage stimulating cytokine.

The experiments described herein (fig. 1) indicate that 406 has the capacity of restoring the weight lost in mice treated with AZT to the normal levels observed in control animals at doses that are 10 to 100 times lower than those previously used with IGF I in mice (Tzarfaty et al., 1994; Pell and Bates, 1992). One of the possible side effects of using large amounts of IGFs in vivo is hypoglycemia, due to their interaction with the insulin receptors. There was no change in the glucose concentrations in mice treated with 14 ng or 140 ng 406. There was an unexpected increase of glucose levels at the dose of 1.4 \(\mu\)g/mouse. The reason(s) for this increase are unknown. It is possible that very high doses of 406 may compete with the binding of insulin to insulin receptors, without actually stimulating glucose uptake. Alternatively, the IL-3 moiety of 406 may be eliciting changes that result in higher glucose concentrations. No published reports were found concerning hyperglycemic effects for IL-3. Nevertheless, tumor necrosis factor-\(\alpha\), which increases in some patients undergoing IL-3 treatment (Seipelt et al., 1993) can cause hyperglycemia (Raina et al., 1997).

The effects of 406 on peripheral blood cells and hematopoietic progenitors reflect the activities described in animal
models and clinical trials for IL-3. 406 increased the number of circulating white cells at levels higher than those observed both in AZT-treated mice and in the control mice receiving water without AZT, and this effect was significant at doses of 14 ng/mouse or higher (figs. 2 and 3). This increase agrees with the elevated number of granulocyte-macrophage colonies in bone marrow and spleen (figs. 4 and 5) as well as the enhanced number of macroscopic colony-forming cells, typical of stem cells with high proliferative potential (McNiece and Bridell, 1994; Pragnell et al., 1994). The generalized increase of white blood cells, and in particular neutrophils and eosinophils, seems to be a typical action on IL-3 in vivo. High doses of IL-3 increased the white cell numbers in HIV type I-infected patients with cytopenia by 50 to 309% (Scadden et al., 1995). Increases in neutrophil counts have also been observed after action of IL-3 administration to patients with aplastic anemia and myelodysplasia (Nimer et al., 1995). There are numerous studies describing combinations of GM-CSF and IL-3 for the treatment of different hematopoietic disorders, for progenitor mobilization and preparation of donors for stem cell transplantation. Nevertheless, there is not a general consensus about the safety and utility of these combinations (Niederwieser et al., 1995; Lemolai et al., 1995; Crump et al., 1993; Nand et al., 1994).

The results obtained with 406 in cells of the erythroid lineage are more complex than those observed with myeloid hematopoietic cells and reflect to a great extent many known effects of IL-3 in vivo. The IL-3 mediated increase of white blood cells described above under conditions of HIV infection, aplastic anemia and myelodysplasia coincides with an increase in the mature megakaryocytic or erythroid cells only in a very limited number of patients, despite the well established multipotent activity in vitro of IL-3 (Scadden et al., 1995; Nimer et al., 1994, Ganser and Hoelzer, 1993). The inadequate erythropoietic response in myelodysplasia and HIV infection could be mediated, in part, by the presence of the erythroid cell inhibitor Tumor necrosis factor-α (Seipel et al., 1993; Kreuzer et al., 1997). It may seem unusual that the high numbers of the erythroid precursors BFU-Es in bone marrow and spleen observed after 406 treatment (Figs. 4 and 5) were observed under conditions in which there was no increase of the average hemoglobin levels (Fig. 2). Nevertheless, there are precedents in the literature indicating that an increase in precursors does not correspond automatically to an increase in mature red cells. IL-3-treated patients with Diamond-Blackman anemia showed an increase in marrow and circulating erythroid progenitors without a concomitant increase in the number of erythrocytes (Bastion et al., 1995). In a similar study involving Diamond-Blackman anemia, only 4 of 18 patients receiving IL-3 had clinically significant improvement in erythroid cells (Gillio et al., 1993). As far as the erythropoietic activity of IL-3 in the presence of AZT is concerned, in vitro studies in cultures of murine spleen and bone marrow (Gogu et al., 1995), retroviral-infected murine bone marrow (Gallicchio and Hughes, 1994) and human bone marrow (Gallicchio and Hughes, 1992; Scadden et al., 1994) have clearly demonstrated the effectiveness of IL-3 in reversing AZT-mediated toxicity of all hematopoietic stem cells including erythroid precursors. The results were particularly striking with the combination of IL-3 and erythropoietin (Gallicchio and Hughes, 1992; Gogu et al., 1995). However, the effects of IL-3 were different in vivo. Although erythropoietin and IL-3 administered alone reduced AZT-mediated anemia in mice treated with low doses of the drug, they were ineffective at high AZT doses. In fact, the combination of IL-3 and erythropoietin at high drug doses increased the AZT-mediated erythroid toxicity (Gallicchio et al., 1993).

These results indicate that the involvement of cytokines in the formation of red cells in vivo is extremely complex. Although erythropoietin is the main factor involved, erythropoiesis can drastically change as a result of an imbalance between stimulatory or inhibitory cytokines. Our studies with the IGF-IL-3 chimera 406 have identified at least one of the apparent causes of the heterogeneous erythropoietic response observed in vivo under AZT-therapy. A careful analysis of the results obtained in experimental groups showing a different degree of anemia indicated that 406 significantly increased the amounts of hemoglobin in those groups showing the highest degree of anemia as compared with those groups with a low degree of anemia (fig. 6). Further experiments under conditions of a pronounced anemic state confirmed the erythropoietic action of 406 at the level of red cell numbers, hematocrit and hemoglobin content (table 2). This experimental system is then ideal for the study of the role played by 406, erythropoietin and other cytokines under conditions of low and high anemia to identify the possible mechanisms behind the heterogeneity of the erythropoietic response often observed after cytokine therapy.

Our results indicate that the fusion protein 406 is very effective in reducing AZT-induced hematopoietic cytotoxicity by reversing completely the weight loss, increasing the number of white blood cells, the number of all hematopoietic precursors and improving the hemoglobin concentrations in mice with severe anemia. 406 should be particularly useful for the recovery of patients with neutropenia. We also hope to define in the future the best conditions for an enhanced

### Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Red Cells (cells/ml × 10³)</th>
<th>Hematocrit (%)</th>
<th>Hemoglobin (g/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal untreated</td>
<td>8.24 ± 0.16</td>
<td>46.6 ± 0.5</td>
<td>142 ± 4</td>
</tr>
<tr>
<td>AZT controls</td>
<td>5.73 ± 0.31</td>
<td>33.0 ± 2.1</td>
<td>102 ± 8</td>
</tr>
<tr>
<td>AZT + 406 (28 ng/mouse)</td>
<td>6.73 ± 0.09</td>
<td>40.3 ± 0.6</td>
<td>119 ± 6</td>
</tr>
</tbody>
</table>

Repeated measures analysis of variance

<table>
<thead>
<tr>
<th>Contrast</th>
<th>P</th>
<th>P</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal unt. vs. AZT</td>
<td>&lt;.001***</td>
<td>&lt;.001***</td>
<td>&lt;.001***</td>
</tr>
<tr>
<td>AZT vs. AZT + 406</td>
<td>&lt;.01**</td>
<td>&lt;.01**</td>
<td>&lt;.05*</td>
</tr>
</tbody>
</table>

*, ** and *** significant at the levels .05, .01 and .001, respectively, Student-Newman-Keuls test.
erythropoietic response. The low cost, large scale production of 406 and similar chimeras between IGFs and cytokines using the baculovirus system will facilitate the design of experiments required to better understand the complex interactions of growth factors and cytokines taking place in vivo.

References


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