In Vivo Hematopoietic Effects of Tumor Necrosis Factor-α in Normal and Erythroleukemic Mice: Characterization and Therapeutic Applications

By Candace S. Johnson, Ming-Jei Chang, and Philip Furmanski

The effects of recombinant, macrophage-derived, murine tumor necrosis factor-alpha (TNF-α) on hematopoiesis in vivo has been examined in normal mice and in Friend virus (FV)-induced erythroleukemic mice. Intravenous (IV) administration of a single dose of recombinant murine TNF-α (10^9 U per mouse) significantly suppressed normal and leukemic late-stage erythropoiesis as measured by numbers of mature erythroid colony forming cells (CFU-E) in the bone marrow and spleen and by peripheral blood reticulocyte counts. In normal animals, the immature erythroid (BFU-E), macrophage (CFU-M), and granulocyte-macrophage (CFU-GM) compartments were significantly stimulated by TNF-α in both the bone marrow and the spleen. In the bone marrow of leukemic mice, the BFU-E, CFU-GM, and CFU-M progenitor cell compartments were also stimulated by treatment with the monokine. In the spleens of leukemic mice (the primary site of FV leukemia cell accumulation), relative numbers of BFU-E and CFU-GM were increased by TNF-α, while those of CFU-M were suppressed. TNF-α caused a rapid decrease in the markedly elevated spleen weights of progressively leukemic mice, and in multiple doses it caused complete clinical disease regression in a significant percentage of leukemic animals. The combination of TNF-α with interferon-gamma (IFN-γ) increased the incidence of leukemia regression, compared with TNF-α alone. These results show that TNF-α exerts a suppressive influence on late-stage erythropoiesis in vivo and suggest that this effect might be exploited in the treatment of acute erythroleukemia, erythroid hyperplasias, and related diseases.

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The model system chosen for these studies is the well-characterized Friend virus (FV)-induced erythroleukemia in mice.1–2 We have shown that macrophages, which play a role in the regulation of normal erythropoiesis,19 and which are the major in vitro source of TNF-α, are centrally involved in the progression and regression of FV erythroleukemia.19,22

We report here that TNF-α suppressed both normal and leukemic late-stage erythropoiesis in vivo and caused a rapid and dramatic decrease in the marked splenomegaly characteristic of progressively leukemic mice. In vivo treatment with TNF-α also exerted significant stimulatory effects on other hematopoietic progenitor cell compartments in both normal and leukemic mice. Multiple-dose treatment with TNF-α caused complete clinical regression in a significant percentage of progressively leukemic mice, and synergistic effects were observed when TNF-α was combined with IFN-γ. These results demonstrate that TNF-α could have therapeutic value in the treatment of some hematopoietic disorders and that therapeutic efficacy may be increased by combination with IFN-γ.

MATERIALS AND METHODS

Viruses. The N-tropic anemia-inducing (CFV) and polycythemia-inducing (FVP) strains of FV were originally obtained from the Department of Cell Biology, AMC Cancer Research Center, Denver.

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Dr C. Friend and Dr E. Mirand, respectively. Viruses were maintained by serial passage of cell-free virus stocks prepared from spleens of leukemic mice (20%, w/vol, in phosphate-buffered saline), as previously described, 23 and stored in sealed ampules at - 70°C. Mice were inoculated intraperitoneally (IP) with 0.5 mL of phosphate-buffered saline containing approximately 100 ID50 of virus. An ID50 is that dose which causes erythroleukemia in 50% of inoculated susceptible mice within 25 days.

Mice. These experiments were performed in inbred Swiss mice termed, National Institutes of Health (NIH)/PLCR mice that were inbred by brother-sister mating. The colonies were regularly monitored for the absence of adventitious viruses. Mice were age- and sex-matched for experimental use and were assessed for percent regression by spleen palpation biweekly in a “blinded” fashion. At the termination of experiments, disease status was confirmed by spleen weight, virus assays, and/or histopathology. 24, 25 For hematopoietic colony assays, spleens or femurs were removed from three to five animals per treatment group and assayed individually. All experiments were replicated at least three times.

Cytokines. Recombinant murine TNF-α and IFN-γ were generously provided by Dr Michael Shepard, Genentech, Inc, South San Francisco. The specific activity for the recombinant TNF-α was 4 × 107 U/mg, determined by use of the LM cell assay performed in serum-free medium. 26 The specific activity for the recombinant IFN-γ was 5 × 106 U/mg, determined by the standard vesicular stomatitis virus assay. 27 All solutions were made with pyrogen-free reagents; the endotoxin content of the TNF-α and IFN-γ used was 0.0836 endotoxin units (EU)/mg and 0.034 EU/mg, respectively.

Erythroid progenitor assays. The plasma clot method of McLeod et al 28 was used for the culture of mature erythroid progenitors (CFU-E). A minimum of three cultures were counted for each assay animal. A modification of the methylcellulose method of Iscove 29 was adapted to agar for the culture of immature erythroid burst-forming progenitors (BFU-E) as follows: 0.25 mL of spleen or bone marrow cells (1 × 105 and 2 × 105 cells per milliliter, respectively) was mixed with 0.25 mL of 10% deionized BSA (containing 0.17% NaHCO3), 0.5 mL erythropoietin (10 U/mL), TCePO, Amgen, Thousand Oaks, CA), 0.4 mL heat-inactivated horse serum, 0.4 mL pokeweed-mitogen-stimulated culture media, 30 0.5 mL heat-inactivated fetal calf serum, 0.2 mL NCTC 109 medium, 0.020 mL hemin (10 mmol/L), 31 and 1.5 mL of an agar mixture containing 1.0 mL of Bacto-Agar (Difco, Detroit) (1.5%), 1.0 mL 2X RPMI 1640, 0.5 mL heat-inactivated fetal calf serum, and 0.050 mL mercaptoethanol (10-2 mol/L). Aliquots of 0.5 mL were plated into the wells of round-bottom well (2.2 x 1.4 cm) culture plates (#76-354-05 Linbro, New Haven, CT). After seven to nine days incubation at 37°C in an N2 atmosphere containing 5% CO2, 5% O2, the agar plugs were fixed with 5% glutaraldehyde, adhered to glass slides, and stained with dimethyloxazin and hematocynin.

Macrophage progenitor assay. Bone marrow or spleen cells were dispersed, washed, centrifuged, and resuspended to a concentration of 2 × 106 or 2 × 107 nucleated cells per millilitre, respectively, in RPMI 1640 plus 15% calf serum. The suspension was diluted tenfold with the same medium containing, in addition, 10% medium conditioned for three days by confluent cultures of L cells, as a source of colony stimulating factor (CSF), and 33% Bacto-Agar. One-milliliter aliquots of the mixture were added to 35-mm plastic bacteriologic dishes and incubated for six days at 37°C in 10% CO2.

Granulocyte-macrophage progenitor assay. Agar cultures were performed as described previously for the CFU-M assay, except that mouse lung-conditioned medium (GM-CSF) 32 was substituted for L-cell conditioned medium as a CSF source. Mouse lung-conditioned medium was prepared by culturing, for 48 hours, whole lung tissue from C57BL/6 mice that were injected three hours previously with 5 µg endotoxin.

Magakaryocyte progenitor assay. Magakaryocytic colony formation was assayed by a modification of the plasma clot culture system described by Clark and Dessypris. 33 Briefly, 0.25 mL nucleated bone marrow or spleen cells (2 × 107 or 2 × 105/mL, respectively) was mixed with 0.45 mL Iscove’s modification of Dulbecco’s medium, 0.375 mL heat-inactivated fetal calf serum treated with charcoal-dextran, 34 0.25 mL of 1% bovine serum albumin, 35 0.025 mL mercaptoethanol (10-4 mol/L), 0.025 mL α-aminocaproic acid (10-3 mol/L), 0.125 pokeweed-mitogen-stimulated spleen cell conditioned medium, 36 0.25 mL erythropoietin (Epo) (10 U/mL, TCePO, Amgen), and 0.25 mL fibrinogen (1.2 mg/mL). Clotting was initiated by the addition of 0.25 mL bovine citrated plasma and 0.25 mL thrombin (2 mL/mL). The plates were then placed (0.5 mL) into the wells of plastic plates (#76-000-05, 2 mL per well capacity; Linbro). After five to six days at 37°C in humidified 5% CO2, clots were harvested, dehydrated on slides, and fixed with glutaraldehyde. The slides were stained for acetylcholinesterase activity and counterstained with hematoxylin. Clusters of three or more acetylcholinesterase-positive cells were counted as colonies.

Platelet counts. Peripheral blood platelet counts were performed according to the method of Brecker and Cronkite. 37

Macrophage XC plaque assay. The quantitation of virus infection of macrophages was performed as previously described. 22

RESULTS

In vivo effect of TNF-α on normal and leukemic erythropoiesis. Treatment of normal bone marrow cells in vitro with TNF-α suppresses CFU-E and BFU-E colony formation. 13-16 In vivo, TNF-α at 1 mg/kg (approximately 7 × 106 U per mouse) has been shown to decrease numbers of erythroid progenitors and, over time, to cause anemia in normal mice. 16 To characterize the effects of TNF-α on erythropoiesis, we tested the in vivo effects of the cytokine on normal mice and animals infected with the CFV and FVP strains of FV. FV erythroleukemia is characterized by proliferation of hematopoietic stem cells in the splenic red pulp, immunosuppression, splenomegaly, hepatomegaly, and death. The CFV strain is associated with mild anemia 39 while the FVP strain is characterized by hypervolemic polycythemia. 32 CFU-E in FVP leukemic mice are not dependent on Epo for colony formation, 39 while CFU-E from CFV-infected animals retain their dependence on Epo for colony formation.

At 14 days postvirus, CFV or FVP progressively leukemic and normal age-matched controls were injected intravenously (IV) with a single dose of 107 units of recombinant murine TNF-α. As shown in Table 1, numbers of CFU-E per femur and spleen were significantly suppressed in both leukemic and normal animals treated with TNF-α. Epo-independent CFU-E, which characterize FVP leukemia, were also substantially suppressed by TNF-α treatment. As previously reported, 19 numbers of CFU-E are increased in the spleens of leukemic mice, but not in the bone marrow.

The effect of TNF-α treatment on late-stage erythropoiesis in leukemic and normal animals was also manifest in peripheral blood reticulocyte counts, which are significantly elevated in FV erythroleukemia. The percentage of reticulocytes (ten to 15 per group) in untreated CFV leukemic mice

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IN VIVO HEMATOPOIETIC EFFECTS OF TNF-\(\alpha\)

Table 1. In Vivo Effect of TNF-\(\alpha\) on CFU-E From Normal and Leukemic Mice

<table>
<thead>
<tr>
<th>Colony Type</th>
<th>Source</th>
<th>Treatment</th>
<th>EPO (0.25 U/mL)</th>
<th>Per Femur (x 10(^{-3}))</th>
<th>%†</th>
<th>Per Spleen (x 10(^{-3}))</th>
<th>%†</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU-E</td>
<td>CFV</td>
<td>None</td>
<td>+</td>
<td>56.7 ± 3.7</td>
<td>7.2%</td>
<td>3,171 ± 768.0</td>
<td>4.1%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TNF-(\alpha)</td>
<td>+</td>
<td>32.1 ± 6.3†</td>
<td>-43</td>
<td>284.5 ± 60.5‡</td>
<td>-91</td>
</tr>
<tr>
<td>FVP</td>
<td>None</td>
<td>+</td>
<td>58.4 ± 6.8</td>
<td>37.8 ± 7.7†</td>
<td>-35</td>
<td>91.9 ± 70.4‡</td>
<td>-96</td>
</tr>
<tr>
<td></td>
<td>TNF-(\alpha)</td>
<td>+</td>
<td>53.7 ± 3.3</td>
<td>23.4 ± 4.7†</td>
<td>-56</td>
<td>104.2 ± 33.3†</td>
<td>-98</td>
</tr>
<tr>
<td>Normal</td>
<td>None</td>
<td>+</td>
<td>56.4 ± 7.5</td>
<td>27.2 ± 6.6†</td>
<td>-52</td>
<td>127.0 ± 20.6†</td>
<td>-40</td>
</tr>
<tr>
<td>BFU-E</td>
<td>CFV</td>
<td>None</td>
<td>+</td>
<td>3.3 ± 0.9</td>
<td>15.1%</td>
<td>89.7 ± 12.9</td>
<td>14.8%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TNF-(\alpha)</td>
<td>+</td>
<td>4.2 ± 0.8§</td>
<td>+27</td>
<td>70.1 ± 13.0§</td>
<td>-22</td>
</tr>
<tr>
<td>FVP</td>
<td>None</td>
<td>+</td>
<td>9.9 ± 2.9</td>
<td>16.2 ± 0.5†</td>
<td>+64</td>
<td>46.7 ± 9.3§</td>
<td>-23</td>
</tr>
<tr>
<td></td>
<td>TNF-(\alpha)</td>
<td>+</td>
<td>3.3 ± 0.6</td>
<td>7.5 ± 1.3†</td>
<td>+127</td>
<td>18.8 ± 1.6†</td>
<td>+86</td>
</tr>
</tbody>
</table>

*Mean ± SD per total femur or spleen was determined in three to five animals that were assayed individually (three cultures counted per animal) three days after IV injection of 10\(^6\) U of TNF-\(\alpha\).
†Percent change from control.
‡Significantly different from control, no treatment (individual animals), \(P < .001\) (Student’s t-test).
§\(P < .01\).

was 14.6 ± 3 (SD), whereas in leukemic mice treated with a single injection of 10\(^3\) U of TNF-\(\alpha\), the percentage was 2.8 ± 1 (\(P < .001\)). Similarly, in normal mice, TNF-\(\alpha\) decreased the percentage of reticulocytes from 7.5 ± 2 to 3.8 ± 2 (\(P < .001\)). Three days after a single injection of 10\(^3\) U of TNF-\(\alpha\), there was no significant decrease in hematocrit in either normal or leukemic mice. However, chronic treatment of normal animals (ten to 15 per group) with TNF-\(\alpha\) (daily injections of 10\(^3\) U for 12 days) resulted in a decrease in hematocrit from 53 ± 2 to 38 ± 4 (\(P < .001\)) by day 12.

To quantitate the response of normal and leukemic late-stage erythropoiesis, animals were injected IV with various doses of TNF-\(\alpha\) and assayed for total CFU-E colony formation in the spleen. As shown in Fig 1, significant suppression was observed in both CFV leukemic and normal mice with a single dose of greater than 10\(^3\) U per mouse. No significant differences were obtained from the in vivo effects of TNF-\(\alpha\) on CFU-E colony formation in normal or CFV leukemic mice. As previously reported,\(^28\) severe side effects (hair ruffling, diarrhea, lethargy) and high mortality were observed in animals treated with 10\(^4\) U of TNF-\(\alpha\).

The time course for suppression of splenic CFU-E in normal animals was determined following a single IV injection of 10\(^2\) U of TNF-\(\alpha\). As shown in Fig 2, numbers of CFU-E were significantly suppressed as early as the day after injection and remained suppressed for up to four days after a single injection of TNF-\(\alpha\). By day 5, numbers of splenic CFU-E returned to normal levels. This time course for CFU-E suppression by TNF-\(\alpha\) was the same in leukemic animals. It should be noted that the serum half-life of TNF is 10.5 minutes.\(^40\) In normal animals, we found that by eight hours after IV injection of 10\(^3\) U of TNF-\(\alpha\), serum levels as measured by the standard LM assay for TNF\(^28\) were undetectable (data not shown).

In contrast to the findings with CFU-E, the immature erythroid (BFU-E) compartments in normal and leukemic mice were stimulated by TNF-\(\alpha\) (Table 1). In the bone marrow, numbers of BFU-E were significantly increased by TNF-\(\alpha\). Similar results were obtained in spleens of normal animals. The spleens of leukemic animals showed a modest decrease in numbers of BFU-E but an increase in their concentration relative to the total number of nucleated cells, reflecting preferential effects of TNF or other splenic cells (CFU-E).

Effects of TNF-\(\alpha\) on macrophage, granulocytic-macrophage, and megakaryocyte progenitors from normal and leukemic animals. TNF-\(\alpha\) decreases in vitro CFU-GM
TNF-α preferentially suppresses late-stage erythroid pro-

and spleen (Table 2). TNF-α induced large increases in
numbers of CFU-M in marrow and spleen of normal
animals. In leukemias, TNF-α treatment increased marrow
CFU-M but suppressed CFU-M numbers in the spleen. As
reported previously,23 CFU-M are productively infected
with virus in progressively FV leukemic mice, and may thus
be more susceptible to suppression by TNF, as shown for other
virus-infected cells in vitro.42,43

Similar results were obtained with CFU-GM (Table 2),
with the exception that numbers of splenic CFU-GM in
leukemic mice remained constant with TNF-α treatment;
this is consistent with the preferential effect of TNF-α on
the massive accumulation of CFU-E in leukemic spleens.

Total peripheral WBC counts and differentials were
unchanged in normal or leukemic animals three days after
TNF-α treatment (data not shown). Whether the increased
numbers of granulocyte/macrophage progenitors in TNF-
α-treated mice are subsequently manifest in increased
peripheral WBCs remains to be determined.

Transient thrombocytopenia has been observed 24 hours
after intramuscular (IM) administration of TNF-α in a
phase 1 clinical trial of cancer patients.11 In these studies, we
have found that three days after TNF-α injection, peripheral
blood platelet counts in untreated normal animals (ten to 15
per group) were 10.1 ± 1.3 (SD) × 10^5/μL, compared with
7.5 ± 0.8 × 10^5/μL in TNF-α-treated animals (P < .001).
The effects of TNF-α on the numbers of CFU-meg were
determined in normal and 14-day leukemic mice. As shown
in Table 2, bone marrow CFU-meg remained unchanged
following TNF-α treatment, whereas splenic CFU-meg were
stimulated. Similar results were obtained when FVP leu-
keemic mice were treated with TNF-α.

TNF-α and regression of leukemia. The finding that
TNF-α preferentially suppresses late-stage erythroid pro-

Table 2. In Vivo Effect of TNF-α on CFU-M, CFU-GM, and CFU-meg From Normal and Leukemic Mice

<table>
<thead>
<tr>
<th>Colony Type</th>
<th>Source</th>
<th>Treatment</th>
<th>No. Colonies*</th>
<th>Per Femur (x 10^-5)</th>
<th>% C†</th>
<th>Per Spleen (x 10^-5)</th>
<th>% C†</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU-M</td>
<td>CFV</td>
<td>None</td>
<td>17.9 ± 4.5</td>
<td>85.5 ± 9.2</td>
<td>-55</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TNF-α</td>
<td>19.8 ± 1.2</td>
<td>38.4 ± 5.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>FVP</td>
<td>None</td>
<td>34.3 ± 4.8</td>
<td>70.8 ± 13.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TNF-α</td>
<td>41.2 ± 2.3</td>
<td>234.4 ± 1.6</td>
<td>-67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>None</td>
<td></td>
<td>20.1 ± 1.5</td>
<td>8.8 ± 2.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TNF-α</td>
<td></td>
<td>42.8 ± 2.8</td>
<td>277.4 ± 5.8</td>
<td>+215</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFU-GM</td>
<td>CFV</td>
<td>None</td>
<td>22.5 ± 7.0</td>
<td>49.4 ± 6.0</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>TNF-α</td>
<td>32.5 ± 8.3</td>
<td>45.4 ± 2.8</td>
<td>-8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>FVP</td>
<td>None</td>
<td>24.7 ± 3.4</td>
<td>26.9 ± 7.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TNF-α</td>
<td>32.0 ± 4.5</td>
<td>30.5 ± 3.5</td>
<td>+13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>None</td>
<td></td>
<td>6.8 ± 0.8</td>
<td>11.5 ± 2.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TNF-α</td>
<td></td>
<td>12.3 ± 3.0</td>
<td>29.2 ± 8.0‡</td>
<td>+154</td>
<td></td>
<td></td>
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<tr>
<td>CFU-meg</td>
<td>CFV</td>
<td>None</td>
<td>1.8 ± 0.4</td>
<td>15.4 ± 3.3</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>TNF-α</td>
<td>2.1 ± 0.6</td>
<td>20.3 ± 3.5‡</td>
<td>+32</td>
<td></td>
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<tr>
<td></td>
<td>FVP</td>
<td>None</td>
<td>1.1 ± 0.4</td>
<td>15.6 ± 2.7</td>
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<tr>
<td></td>
<td></td>
<td>TNF-α</td>
<td>1.2 ± 0.6</td>
<td>22.9 ± 2.3‡</td>
<td>+47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>None</td>
<td></td>
<td>3.5 ± 0.4</td>
<td>6.8 ± 1.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TNF-α</td>
<td></td>
<td>3.2 ± 0.2</td>
<td>19.4 ± 2.0‡</td>
<td>+184</td>
<td></td>
<td></td>
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</table>

*Mean ± SD per total femur or spleen was calculated from three to five animals that were assayed individually (three cultures counted per animal) three days after IV injection of 10^6 U of TNF-α.
†Percent change from control.
‡Significantly different from control, no treatment (individual animals), P < .001 (Student’s t-test).
genitors suggested that this cytokine might have therapeutic value in FV-induced erythroleukemias, a disease characterized by massive accumulation of CFU-E. To determine the effect of TNF-α on the clinical manifestations of leukemia in this system, spleen weights, which are an accurate indicator of leukemic status and viremia in FV-infected animals,23,25 were measured following treatment with TNF-α. Normal and leukemic animals at 14 days postvirus were treated with 10^9 U of TNF-α IV and killed three days later. Spleen weights of CFV and FVP leukemic animals treated with TNF-α were 350 mg ± 100 (SD) and 330 mg ± 100, respectively, whereas spleen weights of untreated leukemic animals were 590 mg ± 70 and 970 mg ± 300 (P < .001). No significant difference was observed in the spleens of normal animals treated (190 mg ± 50) and untreated (180 mg ± 40) with TNF-α.

These decreases in spleen weight observed three days after treatment of leukemic animals with a single dose of TNF-α were transient; all CFV and FVP leukemic animals induced to regress by treatment with TNF-α experienced disease recurrence (spleen weights returning to >500 mg) seven to ten days later.

To determine whether multiple injections of TNF-α would exhibit a greater and more sustained therapeutic effect in this system, progressive CFV leukemic mice at 16 days postvirus inoculation were treated daily for 12 days with TNF-α IP at 10^4 U per injection. Because other studies44,45 have shown that combinations of IFN-γ with TNF-α increase the therapeutic activity of TNF-α, we also tested the effect of addition of IFN-γ (2.5 × 10^8 U per injection) per mouse, given at the same time as TNF-α to the TNF-α regimen. As shown in Fig 3, animals treated with TNF-α alone or TNF-α plus IFN-γ experienced a significant percentage of leukemia regressions (P < .001, χ²); the combination of TNF-α plus IFN-γ caused a significantly higher percentage of regressions than did treatment with TNF-α alone (P < .05). Numbers of regressions in leukemic animals treated with IFN-γ alone were not significantly different from vehicle controls.

Peripheral blood reticulocyte counts and absolute numbers of CFU-E per spleen in animals induced to regress with TNF-α or TNF-α plus IFN-γ also returned to normal values.

We have shown previously that productive virus infection of peritoneal macrophages is an excellent correlate of regression of erythroleukemia.22,23 We therefore determined the infection status of peritoneal macrophages from animals treated with cytokines using the XC plaque assay for infectious virus.22 As shown in Table 3, macrophages from progressively leukemic animals at day 44 postvirus are productively infected with virus. Macrophages from CFV leukemic mice that had been induced to regress temporarily with a single dose of 10^9 U of TNF-α remained productively infected with virus, as did leukemic animals treated with IFN-γ alone. Leukemic animals induced to regress with either multiple doses of TNF-α or TNF-α plus IFN-γ had either very low levels of productive virus infection or no detectable levels of productive virus infection, in accordance with their clinical status (normal spleen weight). Decreasing the frequency of the dose of TNF-α decreased therapeutic efficacy and increased numbers of productively infected macrophages (data not shown).

**DISCUSSION**

Based on hematologic findings in patients with solid tumors undergoing treatment with TNF-α, and studies on the in vitro effects of TNF-α on hematopoietic progenitors and leukemic cells, we considered that this cytokine might have value in the treatment of leukemias and related diseases. The purpose of this report, therefore, was to fully characterize the in vivo effects to murine TNF-α on hematopoietic progenitors in normal and leukemic mice and to evaluate its effects on the clinical manifestations of erythroleukemia in FV-infected animals.

We found that TNF-α is selectively suppressive for late erythroid progenitors (CFU-E) in mice and causes hematologic, virologic, and clinical regression of FV-induced erythroleukemia.

Although the therapeutic index of TNF-α in this system (the quantitative difference in effects on leukemic vs normal cells) was small, treatment with a single dose of the cytokine caused a rapid and massive decrease in the splenomegaly characteristic of the disease and restored, to a considerable degree, the balance among hematopoietic progenitors. Treatment of progressively leukemic mice with multiple doses of TNF-α or combinations of TNF-α with IFN-γ resulted in a significant percentage of permanent disease regressions.

The mechanism of TNF-α effects on erythroid progenitors is unknown. The cytokine may be directly toxic to these cells, it may have additional selective effects against virus-infected cells in leukemic animals, or it may act through other cell...
types. In vitro suppressive effects of TNF-α on CFU-E and BFU-E do not appear to require monocytes or T cells. Because TNF-α had the same effect on CFV (Epo-dependent) and FVP (Epo-independent) leukemic CFU-E, it is unlikely that the cytokine affects erythropoiesis through the primary erythropoietic regulatory hormone, Epo.

TNF has been shown to induce cachexia, which could influence erythropoiesis in several ways. Fasting rats, for example, have extremely low levels of erythropoietin and form the basis for the starved rat erythropoietin assay. However, as noted previously, TNF-α is unlikely to act through effects on Epo. Furthermore, in our studies, mice given a single injection of 10^4 U of TNF-α exhibited no changes in eating habits, and the animals were not noticeably cachetic when killed three days later.

The effects of TNF reported here on various normal hematopoietic compartments have also been observed following in vivo treatment of normal animals with IL-1: IL-1 significantly suppresses CFU-E and stimulates BFU-E, CFU-M, and CFU-GM numbers in vivo. Other studies have demonstrated that in vivo treatment with IL-1 increases numbers of CFU-GM colony formation. TNF also stimulates macrophage production of IL-1, prostaglandin E2, interferon (IFN), GM-CSF, and more TNF. Prostaglandin E2 and interferons have been shown to inhibit erythropoiesis. Thus, TNF-α could thus act alone or through IL-1, or both cytokines could influence hematopoiesis through yet another mediator.

Platelet counts are decreased in patients treated with TNF-α. We found modest decreases in the number of peripheral blood platelets in TNF-α-treated normal animals. TNF has been shown to act on human vascular endothelial cells to induce procoagulant activity, thereby promoting thrombosis. TNF has also been shown to cause endothelial cells to become adhesive for neutrophils. Thus, in TNF-treated animals, peripheral blood platelets may decrease as a result of adherence to endothelial cells.

Spleen CFU-meg colonies were stimulated in both leukemic and normal animals treated with TNF-α. This could be a response to the TNF-induced decrease in platelets. However, the CFU-meg compartment is relatively insensitive to acute perturbations in circulating platelet concentrations. Control of CFU-meg appears to involve a megakaryocyte CSF that is made by activated T cells, although there are reports of both stimulatory and inhibitory effects of T cells and their products on megakaryopoiesis. TNF-α may thus act directly on CFU-meg or indirectly through T cells or their products. No effect of TNF-α was observed on the CFU-meg in bone marrow. The reasons for the difference in TNF sensitivity of spleen and marrow megakaryocyte progenitors remain to be determined. Bone marrow and spleen hematopoietic compartments have been shown to differ in susceptibility to a number of regulatory agents.

These studies were performed using recombinant mouse TNF-α. Native TNF-α appears to lack stringent species specificity. Using recombinant murine and human TNF-α, we (manuscript in preparation) and others have observed quantitative differences in the effects of the cytokines on isologous v homologous cells.

We have previously shown that macrophages play a determinative role in the progression and regression of FV-induced erythroleukemias. We considered, therefore, that macrophages, the primary source of TNF-α, might influence the course of leukemia through their production of this cytokine. Several lines of evidence, however, argue against such a mechanism. First, leukemia regressions are induced by treatment with resting, resident peritoneal macrophages, which produce little TNF-α. In contrast, activated macrophages, which produce large amounts of TNF-α, are incapable of causing regression. Second, macrophage treatment causes regression of CFV- but not FVP-induced erythroleukemias. We found that both CFV and FVP diseases are susceptible to TNF-α. Thus, it would appear that although macrophages are the primary source of TNF-α, and both macrophages and TNF-α induce a rapid, massive decrease in spleen weight and a transient decrease in CFU-E numbers, they may do so by different mechanisms.

It has been shown previously that optimal antitumor activity of TNF-α in 3-methylcholanthrene (MCA) tumor-bearing animals was obtained following treatment with a single injection of 10^5 U of cytokine. In other studies, multiple doses ranging from 10^6 to 1.5 × 10^7 U of TNF were required to induce tumor regression. In FV erythroleukemia, a disease characterized by an increase in virus-infected erythroid precursors, the single high dose (10^7 U) of TNF resulted in profound suppression of CFU-E but did not cause permanent leukemia regression. Disease recurrence in this case is probably due to a failure to eliminate virus cells.
and virus-producing cells. Only multiple doses of TNF over 12 days resulted in disease cures, due to effects on both leukemic erythropoiesis and virus replication.42,43

We found that IFN-γ increased the therapeutic effect of TNF-α in this model system. This effect may be due to the well-known antiviral activity of IFN-γ. But IFN-γ and TNF also act synergistically to kill non-virus-related malignant cells.8,9 Furthermore, IFN-γ is capable of sensitizing target cells to monocyte killing;47 sensitization by IFN-γ may be due in part to its ability to upregulate TNF receptor expression on target cells.48 As described by Balkwill et al,49 the effect of systemic TNF on the regression of human tumor xenografts in nude mice is enhanced by administration of IFN-γ. TNF also stimulates IFN-γ synthesis by monocytes,70 and this action appears to be responsible for the well-known ability of IFN-γ to enhance monocyte cytotoxicity for tumor cells as a result of "macrophage activation factor" activity. Elucidation of the complex interactions between the cytokines is important. It has been suggested that the toxic side effects observed with TNF therapy could be avoided and maximum biologic activity maintained if TNF were given in lower doses in combination with IFN-γ.44,45

In summary, TNF-α selectively suppresses erythropoiesis in vivo and causes clinical regression and restoration of hematologic balance in erythroleukemic mice. The therapeutic efficacy of TNF-α in this system is increased by combination with IFN-γ. These studies form the basis for gaining an understanding of the hematologic effects of TNF-α treatment and provide a responsive model system for further evaluation of TNF-α as a therapeutic agent in certain leukemias and other hematopoietic disorders.

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