We have previously reported that 20 hours' preincubation of human bone marrow cells with interleukin-1β (IL-1) can protect early progenitor cells from 4-hydroperoxycyclophosphamide (4-HC) cytotoxicity. Since tumor necrosis factor-alpha (TNFα) shares many of the biologic properties of IL-1, we have compared the protective effects of TNFα with IL-1 against 4-HC. Incubation of human bone marrow mononuclear cells or an enriched progenitor population for 20 hours with either TNFα or IL-1 resulted in the survival of an increased number of single- and mixed-lineage colonies, including replatable blast cell colonies, while only rare colonies were seen in the control group. Antibodies to TNFα completely abolished the protection observed with IL-1, while antibodies to IL-1α and IL-1β decreased but did not abolish the protection seen with TNFα. Combinations of low doses of TNFα and IL-1 showed synergy in their protective effects. Furthermore, no protection was observed by IL-1, IL-1 bone-marrow-conditioned medium (IL-1-BMCM), or TNFα for HL-60, K562, KG1, KG1α, and DU.528 leukemic-cell lines or primary acute myelogenous leukemic (AML) blast cells from the lethal effects of 4-HC. In the case of HL-60 and KG1α cell lines, TNFα preincubation resulted in increased cytotoxicity. Furthermore, preincubation of a mixture of AML cells and normal bone-marrow cells with IL-1 + TNFα before 4-HC resulted in the protection of normal but not leukemic progenitors. These results suggest that TNFα is necessary for the protection of normal, early, human hematopoietic progenitors from 4-HC, while IL-1 is not mandatory but will synergize with TNFα to offer increased protection. In addition, no protection from 4-HC is observed by TNFα, IL-1, or IL-1-BMCM for primary leukemic blast cells or leukemic cell lines.

MATERIALS AND METHODS

Hematopoietic growth factors. Recombinant human IL-1β (specific activity >2 x 10^7 U/mg) as well as polyclonal rabbit antibodies to human IL-1α and IL-1β were generously supplied by Dr C.A. Dinarello (Boston, MA). These antibodies showed no cross-reactivity with other cytokines, including TNFα. Recombinant human TNFα (specific activity 2.4 x 10^7 U/mg) as well as polyclonal rabbit antibody to TNFα were gifts from Dr Leo Linn, Cetus Corp (Emeryville, CA). The TNFα antibody was shown previously to have no cross-reactivity with IL-1. Recombinant human IL-3 (specific activity 2.3 x 10^8 U/mg) was received as a generous gift from Dr S. Clark, Genetics Institute (Boston, MA). Tissue-culture erythropoietin (TC-Epo; specific activity >10,000 U/mg) was obtained from Amgen (Thousand Oaks, CA). Conditioned medium from the human bladder carcinoma cell line 5637 (5637-CM) was prepared in our laboratory as previously described.

Source of human bone marrow. After obtaining informed consent, bone marrow samples were aspirated from the posterior iliac crest of healthy adult volunteers and placed in tissue culture tubes containing 400 U preservative-free heparin. Mononuclear cells were obtained after centrifugation over Ficoll-Hypaque (FH) (Pharmacia, Upsala, Sweden) for use in our experiments. An enriched population of early hematopoietic progenitors was also used in some experiments.

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experiments. This purified cell population was obtained by introducing the post FH mononuclear cells to elutriation centrifugation containing a Sanderson Chamber and operating at 867g. With increasing flow rates, three fractions of cells based on cell size were collected. The second fraction, which contains the majority of hematopoietic progenitor cells, was further subjected to negative selection by labeling the cells with a panel of monoclonal antibodies (MoAbs), as previously described, followed by the addition of goat antimouse IgG-coated immunomagnetic microspheres. After removal of labeled cells with a magnet, the remaining cells contain an enriched population of hematopoietic progenitors with a plating efficiency of 10% to 20%. All bone marrow cells were kept frozen at −70°C before use.

**Leukemic cells.** Four human leukemic cell lines were obtained from the American Type Cell Collection (ATCC): H1-60, K-562, KG1, and KG1a. H1-60 and K562 leukemic cell lines were maintained in suspension culture in RPMI 1640 with 10% fetal bovine serum (FBS; Hyclone) and KG1 and KG1a in Iscove’s modified Dulbecco medium (IMDM) with 20% FBS. Another human lymphohematopoietic stem-cell line was obtained from Dr. J. Kurtzberg, Duke University Medical Center (Durham, NC) and was maintained as described previously in RPMI 1640 supplemented with 10% horse serum and 10% heat-inactivated FBS, 1 mmol/L pyruvate and 2 mmol/L glutamine. All of the above cell lines were kept under an atmosphere of 5% CO2 in air at 37°C and were used in the logarithmic phase of growth for the experiments described below. Peripheral blood was obtained from newly diagnosed, untreated AML patients with informed consent. These included M1, M2, M3, and M5 types based on the French-American-British (FAB) classification. Mononuclear cells with greater than 90% AML blasts were separated using Ficoll and kept frozen at −70°C as described previously.

**Incubations.** All samples were preincubated for 20 hours as previously described. Briefly, post-FH normal mononuclear cells (5 × 10^6 cells/mL) or an enriched population of hematopoietic progenitors (1.5 × 10^6 cells/mL) were incubated for 20 hours at 37°C in a 5% CO2 humidified atmosphere in the presence of 50 to 100 ng/mL IL-1, 25 to 2500 ng/mL TNFα, IL-1 with 15 μL antibody to TNFα (1:70 dilution, which will neutralize greater than 250 ng/mL TNFα), TNFα with 200 ng/mL antibody to IL-1α and IL-1β (1:50 dilution), and as controls culture medium alone or IL-1 or TNFα with nonspecific rabbit IgG. Studies to evaluate synergy between TNFα and IL-1 were performed by incubating bone marrow cells with suboptimal concentrations of IL-1 (10 ng/mL) and TNFα (0.1 to 1.0 ng/mL). The incubations were performed in polypropylene-coated tubes in an upright position. Each tube contained 4 to 5 mL of mononuclear cell suspension or 1 mL of enriched cell suspension in α-MEM culture medium supplemented with 6% FBS. To assay for any effect on colony-forming cells (CFCs) within the 20-hour preincubation period, bone marrow cells from each different situation were cultured (10^6 cells/mL/dish from bone marrow mononuclear cells and 10^3 cells/mL/dish from the enriched bone marrow cell population) using the same assay as described below.

Leukemic cells (5 × 10^6 cells/mL) were incubated in a similar manner in the presence of 25 ng/mL TNFα, 50% IL-1β (100 ng/mL) bone-marrow-conditioned medium (IL-1–CM), 100 ng/mL IL-1β, or medium alone.

**4-Hydroperoxycyclophosphamide.** 4-HC powder was obtained from Dr. M.O. Colvin, Johns Hopkins Oncology Center (Baltimore, MD). Fresh 4-HC solution in α-MEM was prepared each time just before use. A dose-response curve for 4-HC was determined for each bone marrow in advance of each experiment as follows. After 20 hours of incubation in culture medium without added growth factors, different doses of 4-HC (0 to 120 ng/mL) were added and the incubation continued for an additional 30 minutes at 37°C. The cells were then washed twice with chilled culture medium to remove the 4-HC and cultured in semisolid medium for the growth of hematopoietic CFUs. Colonies were scored weekly for 3 weeks, and the lethal dose was determined as that dose that kills all CFUs except for a few small macrophage colonies. A second sample of the same bone marrow was thawed and incubated with or without the growth factors as described above for 20 hours followed by a 30-minute incubation with the predetermined lethal dose of 4-HC. The cells were then washed twice and cultured using the colony assay as described below. Leukemic cells were treated similarly except that a dose response with 4-HC was performed for each experiment.

**Blast-cell colony assay.** After the 20-hour incubation with or without IL-1β or TNFα, 50 to 100 μg/mL 4-HC was added and the incubation continued for an additional 30 minutes. The cells were washed twice with chilled culture medium to remove the 4-HC and cultured in semisolid medium for the growth of hematopoietic CFUs as previously described. Cultures consisted of α-MEM supplemented with 1.2% methylcellulose (4000 CP, Fisher), 5 × 10^−4 mol/L 2-mercaptoethanol, 1% bovine serum albumin (Sigma Chemical Co, St Louis, MO), 30% FBS, 10^−4 mol/L methylprednisolone sodium succinate, 1 U/mL recombinant human TC-Epo, 50 U/mL IL-3, and 5% 5637-CM. Four to five replicate 1-mL cultures in 35-mm culture dishes (Costar, Cambridge, MA) for each experimental group were maintained in a 5% CO2 humidified atmosphere at 37°C for up to 5 weeks. Colonies were scored on an inverted microscope at weekly intervals for 2 weeks and observed daily thereafter for the appearance of newly formed blast-cell colonies (Bi-CFC). From day 14 on, newly formed blast-cell colonies were picked from the culture dishes with a drawn-out pipette for morphology and replating studies. Cytospin-prepared slides were stained with Wright’s stain to determine the morphology of colonies.

**Replating studies.** In one experiment Bi-CFCs from both IL-1β- and TNFα-treated cultures were picked and replated in 24-well culture plates as previously described. Secondary colonies were scored on days 10 through 14 after replating.

**Assay for leukemic blast progenitors.** The effect of 4-HC on leukemic progenitors was examined using two different assay techniques. First a clonogenic assay was performed for the leukemic cell lines HL-60, K562, KG1, and primary peripheral-blood acute myelogenous leukemia (AML) blast cells. In this assay cells were cultured in α-MEM with 2% methylcellulose and 25% FBS. Five percent 5637-CM was added to all cultures of AML blasts and KG1 cells. Triplicate 1-mL cultures with different cell concentrations (10^3 to 10^5 cells of the leukemic cell lines and 5 × 10^4 to 5 × 10^5 AML blast cells) per plate were prepared for each experimental condition. Colonies (greater than 20 cells) were scored using an inverted microscope at day 7 of culture. In addition, blast cells obtained from the peripheral blood of an AML patient and capable of growing in a clonogenic assay were used for mixing experiments with post FH normal bone marrow cells. Post FH normal bone marrow with or without 17% AML cells were incubated for 20 hours with medium alone or 10 ng/mL IL-1 + 5 ng/mL TNFα. 4-HC (65 μg/mL) was added for 30 minutes followed by washing the cells twice with cold medium. The cells were then cultured in the blast-cell colony assay as described above. Leukemic clusters (CFU-L) of more than 3 cells as well as normal colonies were scored weekly. CFU-L clusters were readily identified in situ and confirmed morphologically by Wright’s stained cytopsin-prepared slides. A second assay was used for the nonclonogenic leukemic cell lines DU-528 and KG1a. After 4-HC treatment, 2 × 10^6 cells in doubling dilutions were cultured in triplicate in 96-well plates for 20 hours in 200 μL of medium. [3H]-thymidine (1 μCi per well) was added and the incubation continued for another 4 hours. Cells were harvested using a microplate cell harvester (Cambridge Technology, Inc, Watertown, MA) and treated as described above.
MA) and the incorporation of [H]-thymidine recorded. Counts from control versus 4-HC–treated cultures were compared to determine whether IL-1 or TNFα offered any protection for leukemic cells from the chemotherapeutic agent 4-HC.

**TNFα ELISA.** Supernatants from different bone marrows incubated with or without 100 ng/mL IL-1 for 20 hours were collected and assayed for TNFα levels using a TNFα enzyme-linked immunosorbent assay (ELISA) kit purchased from R&D Systems Inc (Minneapolis, MN). The assay is capable of detecting as low as 20 pg/mL TNFα.

**Statistical analysis.** Three to six replicates of each experiment were performed. Levels of significance for comparison between samples in each experiment were determined using the two-tailed Student’s t test. The results are expressed as means ± 1 SD of three to five plates per point.

**RESULTS**

We have previously shown that preincubation for 20 hours with IL-1 before 4-HC treatment results in a significant increase in detectable single and multilineage colonies including B1-CFC.17 In the present study we have compared the ability of TNFα and IL-1 preincubation for their ability to protect these early bone-marrow progenitors from 4-HC. Table 1 shows that preincubation of bone marrow cells with TNFα for 20 hours also protects a significant number of CFCs from 4-HC. In fact, TNFα at a dose twofold lower than that of IL-1 was able to protect as many CFCs as IL-1 when measured at day 21 of culture. To determine whether the increase of CFCs by TNFα was due to the preservation of specific lineage, the morphology of colonies developing was determined. As shown previously17 for IL-1, a 20-hour incubation with IL-1 or TNFα in the absence of 4-HC treatment results in a nonsignificant variable effect on colony formation when compared with bone marrow incubated for 20 hours in the absence of IL-1 or TNFα. Table 1 also demonstrates that the type of colonies as well as the percentage of total CFCs protected by TNFα preincubation were very similar to the type of colonies seen with IL-1. About 4% of the CFCs were protected in the experiment shown in Table 1. This probably represents a minimum recovery as calculated at day 21 of culture, since more colonies continue to appear later in culture. In general the average recovery from three different experiments was 2% to 6%. Interestingly, higher doses of TNFα result in an absolute decrease in protection when compared to control. However, when compared with the colonies formed pre–4-HC, very little difference in the percentage recovery is seen. This is because the number of colonies formed pre–4-HC in the presence of increasing concentrations of TNFα also decreases. The type of colonies most affected appeared to be those containing erythroid cells (ie, BFU-E and CFU-Mix). A similar inhibition by TNFα on CFCs has been previously reported,27,34 suggesting that high doses of TNFα in our experiments might be toxic to early progenitor cells. The results of replating studies are shown in Table 2 and demonstrate that replating of B1-CFC from either IL-1 or TNFα pretreated cultures gave similar results with the appearance of secondary colonies consisting of macrophages, granulocytes and macrophages, eosinophils, erythroid cells, mixed granulocyte/macrophage/erythroid/eosinophils, and undifferentiated blast colonies. The plating efficiencies for both were about 20%.

Since TNFα and IL-1 are capable of inducing each other,10-14 we studied the protection effect of each cytokine for early progenitors from 4-HC in the presence of polyclonal rabbit antibodies to the other factor. Figure 1 shows the results of two representative experiments of five in which the IL-1 protective effect was completely abolished when bone marrow cells were preincubated with IL-1 in the presence of antibody to TNFα. The effect of TNFα antibodies was specific, since an equal volume of nonspecific rabbit IgG showed no effect, and no effects were seen when bone marrow cells were cultured with antibodies to TNFα before 4-HC treatment (data not shown). In contrast, preincubation of bone marrow cells with TNFα in the presence of antibody to IL-1α and IL-1β resulted in decreased protection of early progenitors (Fig 2). However, the protection was not completely abolished, as seen for IL-1 plus anti-TNFα (Fig 1). Again, this effect of antibodies to IL-1 was specific, since controls performed with nonspecific rabbit IgG showed no effect.

In support of the above results, we measured the amount of endogenous TNFα present in supernatants obtained from bone marrow cultures incubated with 100 ng/mL IL-1 for 20 hours. Table 3 demonstrates the detection of a significant amount of TNFα as measured by ELISA in the supernatant.

---

**Table 1. Effects of 20 Hours' Preincubation With Recombinant IL-1 and TNFα on Different Colony Types After Treatment With 4-HC**

<table>
<thead>
<tr>
<th>Type of Colonies</th>
<th>Control</th>
<th>IL-1 (50 ng/mL)</th>
<th>TNFα (25 ng/mL)</th>
<th>TNFα (250 ng/mL)</th>
<th>TNFα (2500 ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>1 ± 1 (0.2)</td>
<td>33 ± 4 (10)</td>
<td>31 ± 4 (6.9)</td>
<td>41 ± 7 (10.5)</td>
<td>23 ± 3 (7.7)</td>
</tr>
<tr>
<td>GM</td>
<td>0 (0)</td>
<td>17 ± 2 (5)</td>
<td>12 ± 6 (3.3)</td>
<td>20 ± 7 (11.1)</td>
<td>6 ± 1 (10.0)</td>
</tr>
<tr>
<td>Eo</td>
<td>0 (0)</td>
<td>1 ± 1 (0.5)</td>
<td>2 ± 1 (0.8)</td>
<td>2 ± 3 (1.0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>E</td>
<td>3 ± 4 (0.15)</td>
<td>107 ± 12 (3.5)</td>
<td>97 ± 20 (3.4)</td>
<td>55 ± 10 (2.3)</td>
<td>4 ± 3 (0.5)</td>
</tr>
<tr>
<td>Mix</td>
<td>0 (0)</td>
<td>18 ± 4 (3.3)</td>
<td>25 ± 3 (4.6)</td>
<td>19 ± 3 (5.7)</td>
<td>2 ± 1 (2.2)</td>
</tr>
<tr>
<td>B1</td>
<td>0 (0)</td>
<td>17 ± 6 (9.4)</td>
<td>33 ± 10 (27.5)</td>
<td>31 ± 14 (25.8)</td>
<td>9 ± 4 (30.0)</td>
</tr>
<tr>
<td>Total</td>
<td>4 ± 4 (0.13)</td>
<td>182 ± 20 (4.2)</td>
<td>180 ± 14 (4.2)</td>
<td>157 ± 7 (4.4)</td>
<td>38 ± 3 (3.0)</td>
</tr>
</tbody>
</table>

---

The data represent the mean ± 1 SD of the number of colonies obtained in four to five replicate culture plates on day 21 of culture with each culture plate containing 3 × 10^5 cells from an enriched bone-marrow cell population. Values in parentheses represent the percent of recovery of CFCs at day 21 after 4-HC treatment in comparison with total CFCs present in the absence of 4-HC.

Abbreviations: GM, granulocyte/macrophage; M, macrophage; Eo, eosinophil; E, erythroid burst; Mix, mixed colonies containing erythroid, granulocyte, macrophage, megakaryocyte, and/or eosinophil; B1, blast cell colony.

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of four different bone marrows incubated with IL-1, whereas little or no detectable TNFα activity was seen in the supernatants of three of these bone marrow incubated in the absence of IL-1.

Next we determined whether IL-1 and TNFα would provide additive or synergistic protection for hematopoietic progenitor cells by incubating bone marrow cells with suboptimal doses of IL-1 and TNFα before 4-HC treatment. Table 4 demonstrates that combining IL-1 and TNFα in the preincubation results in a synergistic increase in protection from 4-HC. The combination of 10 ng/mL IL-1 with 1 ng/mL TNFα results in 161 colonies at day 21 after 4-HC, whereas 10 ng/mL IL-1 alone gave only a mean of four colonies, and 1 ng/mL TNFα alone resulted in a mean of 20 colonies. Similarly, combining 10 ng/mL IL-1 with 0.1 ng/mL TNFα in a second experiment resulted in greater than 300 colonies, while IL-1 alone gave 116 colonies and TNFα alone gave 40 colonies. Similar synergy was observed in a third experiment in which an enriched progenitor cell population was used. These results are consistent with a synergistic action of IL-1 and TNFα on the protection of normal early hematopoietic progenitor cells from the effects of 4-HC.

**Table 2. Results of Replating Studies From TNFα- and IL-1–Pretreated Cultures**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day of Harvest*</th>
<th>M</th>
<th>GM</th>
<th>Eo</th>
<th>E</th>
<th>Mix</th>
<th>B1</th>
<th>Plating Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>22</td>
<td>14</td>
<td>27</td>
<td></td>
<td>2</td>
<td>3</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>9</td>
<td>2</td>
<td></td>
<td>2</td>
<td>3</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>7</td>
<td>21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>8</td>
<td>18</td>
<td></td>
<td>1</td>
<td>4</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>9</td>
<td>3</td>
<td></td>
<td>2</td>
<td>3</td>
<td>19</td>
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</tr>
<tr>
<td></td>
<td>27</td>
<td>10</td>
<td>11</td>
<td></td>
<td>8</td>
<td>3</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>+TNFα</td>
<td>22</td>
<td>7</td>
<td>3</td>
<td></td>
<td>2</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
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<td>7</td>
<td>2</td>
<td>26</td>
<td>9</td>
<td>5</td>
<td>27</td>
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<td>9</td>
<td>9</td>
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<td>18</td>
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<td></td>
<td>22</td>
<td>12</td>
<td>1</td>
<td>15</td>
<td>5</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>16</td>
<td>7</td>
<td>1</td>
<td>1</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>9</td>
<td>3</td>
<td></td>
<td>2</td>
<td>4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*On days 22 and 27, a total of 10 and 12 blast-cell colonies were picked for replating from each of two culture dishes of IL-1 and TNFα-pretreated groups, respectively.

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**Fig 1.** The effect of 20 hours preincubation with (A) culture medium alone, (B) IL-1 plus nonspecific rabbit IgG, or (C) IL-1 plus antibodies to TNFα on the recovery of CFCs from 4-HC–treated bone marrow mononuclear cells. The results of two experiments are shown and expressed as the mean ± 1 SD of the total number of colonies scored in four to five replicate culture dishes. In experiment 1, 5 × 10⁸/mL bone-marrow mononuclear cells were treated with 50 ng/mL IL-1, 65 μg/mL 4-HC, and colonies counted at day 21. In experiment 2, an enriched progenitor cell population containing 1.5 × 10⁶ cells/mL were treated with 100 ng/mL IL-1, 80 μg/mL 4-HC, and colonies counted at day 28. In both experiments a highly significant difference was seen comparing the IL-1 group (B) with the control (A) or IL-1 + anti-TNF group (C) (P < .005).

**Fig 2.** The effect of 20 hours preincubation with (A) culture medium alone, (B) TNFα plus nonspecific rabbit IgG, or (C) TNFα plus antibodies to IL-1α and IL-1β on the recovery of CFCS from 4-HC–treated bone marrow mononuclear cells. The results of two experiments are shown and expressed as the mean ± 1 SD of the total number of colonies scored in four to five replicate culture dishes. In experiment 1, 1.5 × 10⁸ cells/mL from an enriched progenitor cell population were treated with 25 ng/mL TNFα, 60 μg/mL 4-HC, and colonies counted at day 28. In experiment 2, 5 × 10⁸/mL bone marrow mononuclear cells were treated with 5 ng/mL TNFα, 70 μg/mL 4-HC, and colonies counted on day 21. In both experiments the protection with TNFα (B) was significantly greater when compared with medium alone (A) or TNFα + antibodies to IL-1α and IL-1β (C). In addition, the protection seen with TNFα + antibodies to IL-1α and IL-1β (C) was still significantly greater than that seen with medium alone (A; P < .025).
For the protection from 4-HC observed by IL-1 and TNFα to have clinical relevance, especially in the setting of purged autologous bone marrow transplantation, it must be shown that IL-1 and TNFα provide no protection from 4-HC for leukemic cells. To accomplish this we studied the effects of 20 hours preincubation with IL-1, TNFα, or conditioned medium obtained from bone marrow cells incubated with IL-1 for 20 hours (IL-1-BMCM) on five different leukemic cell lines and on five samples of leukemic blast cells from the peripheral blood of five AML patients with greater than 90% blasts. Figure 3 shows the results of the effects of 20 hours preincubation with medium, TNFα, or IL-1-BMCM on colony formation by HL-60 leukemic cells in the presence of increasing concentrations of 4-HC. No protection is seen for HL-60 colony formation with TNFα or IL-1-BMCM at any of the 4-HC concentrations tested. In fact, TNFα alone in the absence of 4-HC resulted in a significant increase in inhibition of the number of colonies obtained when compared with the control medium group (P < .05). Similar results were seen for K562, KG1, and DU.528 leukemic cells, although TNFα in the absence of 4-HC showed no effect (data not shown). Similarly, using [3H]-TdR incorporation as a measure of cell proliferation, no protection was observed for TNFα or IL-1-BMCM when compared with control for KG1a leukemic cells in the presence of increasing concentrations of 4-HC (Fig 4). Once again TNFα and IL-1-BMCM alone significantly suppressed proliferation of KG1a cells when compared with the medium control (P < .025).

In addition, we tested the effects of 20 hours preincubation with IL-1, TNFα, or IL-1-BMCM before 4-HC on leukemic blast-cell colony formation obtained from patients with AML. Figure 5 shows the results of one of five representative AML patients studied. No effect on leukemic colony formation was seen with any of the materials tested. Increasing concentrations of 4-HC reduced the number of leukemic colonies scored, and prior incubation with IL-1, TNFα, or IL-1-BMCM showed no effect. Similar results were seen with the other four AML samples, which contained leukemic blasts from AML patients classified as M1, M2, and M5 using the FAB classification.

Finally, leukemic blast cells from a sixth AML patient (classified as M3, HLA-DR−, TDT+) were used for mixing experiments with normal bone marrow. The mixed cell suspension contained 17% AML cells. Preincubation with 10 ng/mL IL-1 + 5 ng/mL TNFα protected normal colony formation when compared with medium alone whether the normal cells were cultured alone or mixed with leukemic cells (Table 5). No leukemic cluster formation was observed after 4-HC treatment whether the leukemic cells were incubated in the presence of normal marrow with medium alone or IL-1 + TNFα (Table 5). Similar protection from 4-HC of normal but not leukemic progenitors was observed when the mixture was preincubated with IL-1 or TNFα alone (data not shown).

Table 4. Effect of Preincubation With IL-1, TNFα, or IL-1 + TNFα on Protection of Early Hematopoietic Progenitor Cells From 4-HC

<table>
<thead>
<tr>
<th>Day of Culture</th>
<th>Control</th>
<th>IL-1 (10 ng/mL)</th>
<th>0.1 ng/mL</th>
<th>1.0 ng/mL</th>
<th>IL-1 + TNFα</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp 1 Day 21</td>
<td>3 ± 1</td>
<td>4 ± 4</td>
<td>20 ± 10</td>
<td>161 ± 28</td>
<td></td>
</tr>
<tr>
<td>Exp 2 Day 14</td>
<td>2 ± 1</td>
<td>71 ± 14</td>
<td>24 ± 10</td>
<td>232 ± 59</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 ± 1</td>
<td>116 ± 16</td>
<td>40 ± 8</td>
<td>&gt;300</td>
<td></td>
</tr>
<tr>
<td>Exp 3 Day 21</td>
<td>0.5 ± 0.6</td>
<td>3 ± 1</td>
<td>30 ± 3</td>
<td>71 ± 23</td>
<td></td>
</tr>
</tbody>
</table>

In experiments 1 and 2, post-Ficoll-Hypaque bone-marrow mononuclear cells were used. In experiment 3, an enriched bone-marrow cell population was used. The 4-HC dose used in experiments 1 and 2 was 70 μg/mL, while 65 μg/mL 4-HC was used in experiment 3. The results are expressed as mean ± SD of total number of colonies in four to five replicate culture dishes.

Fig 3. Effect of 20 hours preincubation with 50% IL-1-BMCM, TNFα (25 ng/mL), or culture medium alone on the recovery of HL-60 leukemic colonies from 4-HC. The results of one of three similar experiments is shown. The values shown represent the mean ± 1 SD of the total number of colonies counted on day 7 of three replicate culture dishes seeded with 10⁴ to 10⁵ cells per dish. The inhibition seen with TNFα was significant (P < .025) when compared to the medium alone group.

Table 3. TNFα Levels in IL-1-Bone Marrow Conditioned Medium

<table>
<thead>
<tr>
<th>Cultures</th>
<th>+ IL-1 (pg/mL)</th>
<th>- IL-1 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>85</td>
<td>&lt;20</td>
</tr>
<tr>
<td>2</td>
<td>97</td>
<td>&lt;20</td>
</tr>
<tr>
<td>3</td>
<td>180</td>
<td>&lt;20</td>
</tr>
<tr>
<td>4</td>
<td>150</td>
<td>—</td>
</tr>
</tbody>
</table>

Levels measured by TNFα ELISA kit, sensitivity greater than 20 pg/mL. Conditioned medium from four different post-Ficoll-Hypaque bone marrow cells incubated for 20 hours with 100 ng/mL IL-1 were analyzed.
matopoietic progenitor cells including replatable B1-CFC from 4-HC (Table 1). In general, TNFα was shown to be more potent than IL-1 in terms of protecting CFCs from 4-HC demonstrating protective effects with doses as low as 0.1 ng/mL while IL-1 becomes unpredictable at low concentrations (ie, 10 ng/mL [Table 4]). Higher doses of TNFα displayed significantly less protection (Table 1). This decrease in protection for TNFα from 4-HC correlates with the TNFα inhibitory effect on CFCs reported previously.27-31 and seen in this study following 20 hours incubation with TNFα (Table 1). All types of colonies were inhibited. In spite of this inhibitory effect, it is clear that a wide range of TNFα concentrations can be used to demonstrate significant protection from 4-HC (Tables 1 and 4).

Because of previous reports regarding the ability of IL-1 or TNFα to induce synthesis of each other,10-14 we have investigated the following possibilities: (1) Is the IL-1 protection mediated by TNFα? (2) Does TNFα act indirectly via IL-1 production? or (3) Does TNFα act similarly and/or independently of IL-1 to provide protection against 4-HC toxicity? Our results show that antibodies to TNFα can abolish completely any protection seen by IL-1 (Fig 1). Furthermore, a significant amount of TNFα was measured in the supernatants of bone marrow cells incubated for 20 hours with IL-1 while none was detected in the supernatants of bone marrow cells incubated for 20 hours with IL-1-1 BMCM. As shown in Table 3, although these results suggest that TNFα is the mediator of the protection provided by IL-1, the addition of IL-1α and IL-1β antibodies to cells incubated with TNFα resulted in a significant decrease (P < .01) but not elimination of the TNFα protective effect (Fig 2). Taken together these studies suggest that TNFα is a necessary factor for the protection from 4-HC toxicity and that IL-1 interacts with TNFα to increase that protection. Support for this TNFα–IL-1 interaction comes from the results of our synergy studies. We have incubated bone marrow cells with low doses of combinations of IL-1 and TNFα prior to treatment with 4-HC. A synergistic effect on the number of CFCs recovered was observed when 0.1 to 1.0 ng/mL TNFα was mixed with 10 ng/mL IL-1 in three different experiments. Synergy between IL-1 and TNFα has been shown previously in different biologic activities.38-40 To date no synergistic effect between IL-1 and TNFα has been reported for the protection of hematopoietic cells from chemotherapeutic agents or in radioprotection. An additive effect was reported when both human TNFα and IL-1 were injected into mice 20 hours before lethal irradiation.41 The mechanism for such synergy is not known. Because our target bone-marrow population is heterogeneous, we cannot rule out the possibility that the protection displayed by IL-1 and/or TNFα in the present study is indirect through the effects of these cytokines on accessory cells. Because IL-1 and TNFα do not share any apparent homology nor recognize the same cellular receptor, and since IL-1 down regulates the TNFα receptor42 as well as its own43 while TNFα has no influence on the IL-1 receptor, we speculate that the apparent synergy may result from similar effects on the postreceptor biochemical events that are not yet well defined. Another possible reason for the synergistic effect reported

DISCUSSION

We have recently reported that 20 hours preincubation of normal human bone marrow cells with IL-1 can protect early progenitors from the lethal effects of high doses of 4-HC.17,26 Since IL-1 is known to induce the production of other cytokines from accessory cells and because we used a relatively crude population of cells in our previous experiments, we have investigated whether the protective effect observed with IL-1 is a direct effect of the IL-1 or an indirect effect through the production of other cytokines during the 20-hour incubation. We have previously reported that 20 hours preincubation with IL-6 or IL-3 did not protect human progenitor cells from 4-HC. In fact, use of these cytokines may increase the toxicity of 4-HC.26 In the present study we report that TNFα is also capable of protecting early hematopoietic progenitors from the lethal effects of high doses of 4-HC.17,26 In general, TNFα was shown to be more potent than IL-1 in terms of protecting CFCs from 4-HC demonstrating protective effects with doses as low as 0.1 ng/mL while IL-1 becomes unpredictable at low concentrations (ie, 10 ng/mL [Table 4]). Higher doses of TNFα displayed significantly less protection (Table 1). This decrease in protection for TNFα from 4-HC correlates with the TNFα inhibitory effect on CFCs reported previously.27-31 and seen in this study following 20 hours incubation with TNFα (Table 1). All types of colonies were inhibited. In spite of this inhibitory effect, it is clear that a wide range of TNFα concentrations can be used to demonstrate significant protection from 4-HC (Tables 1 and 4).

Because of previous reports regarding the ability of IL-1 or TNFα to induce synthesis of each other,10-14 we have investigated the following possibilities: (1) Is the IL-1 protection mediated by TNFα? (2) Does TNFα act indirectly via IL-1 production? or (3) Does TNFα act similarly and/or independently of IL-1 to provide protection against 4-HC toxicity? Our results show that antibodies to TNFα can abolish completely any protection seen by IL-1 (Fig 1). Furthermore, a significant amount of TNFα was measured in the supernatants of bone marrow cells incubated for 20 hours with IL-1 while none was detected in the supernatants of bone marrow cells incubated for 20 hours with IL-1-1 BMCM. As shown in Table 3, although these results suggest that TNFα is the mediator of the protection provided by IL-1, the addition of IL-1α and IL-1β antibodies to cells incubated with TNFα resulted in a significant decrease (P < .01) but not elimination of the TNFα protective effect (Fig 2). Taken together these studies suggest that TNFα is a necessary factor for the protection from 4-HC toxicity and that IL-1 interacts with TNFα to increase that protection. Support for this TNFα–IL-1 interaction comes from the results of our synergy studies. We have incubated bone marrow cells with low doses of combinations of IL-1 and TNFα prior to treatment with 4-HC. A synergistic effect on the number of CFCs recovered was observed when 0.1 to 1.0 ng/mL TNFα was mixed with 10 ng/mL IL-1 in three different experiments. Synergy between IL-1 and TNFα has been shown previously in different biologic activities.38-40 To date no synergistic effect between IL-1 and TNFα has been reported for the protection of hematopoietic cells from chemotherapeutic agents or in radioprotection. An additive effect was reported when both human TNFα and IL-1 were injected into mice 20 hours before lethal irradiation.41 The mechanism for such synergy is not known. Because our target bone-marrow population is heterogeneous, we cannot rule out the possibility that the protection displayed by IL-1 and/or TNFα in the present study is indirect through the effects of these cytokines on accessory cells. Because IL-1 and TNFα do not share any apparent homology nor recognize the same cellular receptor, and since IL-1 down regulates the TNFα receptor42 as well as its own43 while TNFα has no influence on the IL-1 receptor, we speculate that the apparent synergy may result from similar effects on the postreceptor biochemical events that are not yet well defined. Another possible reason for the synergistic effect reported
here is that TNFα and IL-1 may exert their effects at the level of transcription and stabilization of certain messenger RNAs (mRNAs) necessary for the protection from 4-HC. TNFα may increase the transcription rate of these putative mRNAs, and IL-1 may stabilize the message so that the end product is much higher in the presence of both factors, whereas TNFα alone will still provide protection and IL-1, in the absence of TNFα, will not.

The findings that TNFα and IL-1 are able to protect normal hematopoietic progenitor cells from 4-HC may have clinical significance if their protective effect is selective for normal cells when compared to malignant cells. We have previously reported the lack of protection for HL-60 and K562 leukemic cell lines from 4-HC when preincubated with IL-1 or IL-1–BMCM as well as the protection of normal but not HL-60 cells in mixing experiments. In the present study we compared the effects of 20 hours incubation with IL-1–BMCM, IL-1, or TNFα on the toxicity of 4-HC for human leukemic cell lines as well as AML blast cells. We have used both clonal assays and 3H-TdR uptake studies to measure proliferation. Our results demonstrate that IL-1β, IL-1–BMCM, or TNFα do not protect HL-60, K562, KG1, KG1a, or DU.528 leukemic cell lines or any of five different AML blast cell populations from the lethal effects of 4-HC. In fact, significant increased toxicity to 4-HC was observed against HL-60 and KG1a cells merely by the preincubation with IL-1–BMCM or TNFα. Furthermore, in a mixing experiment we have shown that IL-1 + TNFα can protect normal hematopoietic progenitors but not AML progenitors from the cytotoxicity of 4-HC. Among the AML blast-cell populations tested were at least four different FAB classes (M1, M2, M3, and M5).

Tumoricidal activity against certain leukemic as well as solid tumors has been reported for IL-135–38 as well as TNFα. In addition, TNFα was reported to synergize with some chemotherapeutic agents in vivo and in vitro to kill tumor cells.63–65 A recent in vivo study showed that IL-1 protects mice from lethal irradiation but has no effect on tumors in the same animals.66 Thus far our results suggest that the protection by TNFα and IL-1 may be selective for normal hematopoietic progenitors and may mediate additional antitumor effects together with 4-HC in certain leukemias. Although IL-1 receptors were found on AML cells67 but not on HL-60, KG1, or K562 cells,68 TNFα receptors were found on AML as well as HL-60, KG1, KG1a, and K562 cells.69,70 Since TNFα appears to be the dominant factor involved in the protection of normal progenitors, the selectivity by TNFα may be attributable to the phenomena of reverse signal transduction first described by Hatakeyama et al.71 These authors pointed out that growth-factor receptor interactions may induce the reverse effect on neoplastic cells when compared with normal cells. However, no direct experimental evidence is available for this using TNFα or IL-1, and alternative explanations are possible.

In summary, we have shown that TNFα may be the dominant cytokine responsible for the protection of early human progenitors from 4-HC. IL-1 was shown to synergize with TNFα and to enhance such protection. So far there is no evidence for similar protection for leukemic progenitors by TNFα and IL-1. The clinical significance of these findings is being tested in our laboratory using an animal model system. Other in vitro studies are also being conducted to investigate the possible mechanisms responsible for the protection of these primitive progenitors by TNFα and/or IL-1.

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