Ferritin Stimulation of a Monokine Inhibitor of Lipopolysaccharide-Augmented Myelopoiesis Is Ferroxidase Dependent

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Ferritin inhibition of myelopoiesis has been associated with intrinsic ferroxidase activity of heavy-chain ferritin and with production of a monokine inhibitor of lipopolysaccharide (LPS)-augmented monocytopoiesis. We report here that intrinsic ferroxidase activity of heavy-chain ferritin is required for stimulated production of the monokine inhibitor of LPS-augmented monocytopoiesis.

Heavy-chain, acidic ferritin (H-ferritin), but not light-chain, basic ferritin (L-ferritin), is a negative regulator of myelopoiesis for humans and mice (1–3, 6–10). H-ferritin, which is produced by inflammatory macrophages (3, 5), inhibits colony-stimulating factor (CSF)-dependent clonal proliferation and differentiation of major histocompatibility class II antigen-rich progenitors in the S phase of the cell cycle (1, 16). Investigations using murine marrow and rat liver ferritin have identified the sensitive mononuclear phagocyte progenitors as pre-lineage commitment progenitors which are incapable of responding solely to the lineage-specific growth factor macrophage CSF (M-CSF) (17, 18). The progenitors require an additional nonspecific factor such as bacterial lipopolysaccharide (LPS) to become responsive to the growth factor (17, 18), and further investigation has implicated tumor necrosis factor alpha (TNF-α) as the mediator of the costimulatory effect of LPS (12). In addition, we have recently reported that ferritin inhibition of two-signal-dependent colony formation is cytokine mediated rather than a direct effect. In combination with M-CSF, ferritin stimulates macrophages to produce an as-yet-identified monokine that inhibits LPS- or TNF-α-augmented in vitro M-CSF-dependent monocytopoiesis (11). Myelopoiesis is regulated by interacting cytokines, and the opposing influences of TNF-α and the ferritin-induced monokine may be important in regulating the rate of cell lineage commitment and ultimately new mononuclear phagocyte generation during infectious or inflammatory processes.

H-ferritin inhibition of myelopoiesis has been linked to intrinsic ferroxidase activity, i.e., the ability to catalyze the oxidation of ferrous iron to ferric iron (4, 13, 14). The present investigation was performed to determine if ferroxidase activity is similarly required for stimulated production of the inhibitory monokine. Figure 1 shows a schematic representation of the envisioned stimulatory and inhibitory steps in monocytopoiesis which this investigation was designed to assess. In our initial experiments, ferroxidase-positive, recombinant human ferritin and a recombinant ferroxidase-negative mutein (4) were compared for inhibitory effect in murine marrow cultures stimulated with highly purified murine M-CSF (specific activity > 5 × 10⁷ U/mg of protein) (17, 18). The recombinant ferritins were provided by Paolo Arosio and Sonja Levi, University of Milan, Milan, Italy, and their preparation and characterization have been described previously (14). The mutein (no. 222) was produced by oligonucleotide-site-directed mutagenesis, with substitutions of Glu, Lys, and Gly, respectively, for Lys-86, Glu-62, and His-65 abolishing ferroxidase activity but leaving the outer portions of the molecule unaffected (13–15). The soft-agar culture we used for enumeration of mononuclear phagocyte progenitors has been described in detail (17, 18). Escherichia coli O111:B4 LPS at 0.1 ng/ml was used as the costimulant with M-CSF for stimulation of two-signal-dependent colony formation (17, 18). As shown in Table 1, addition of LPS increased the colony yield by approximately 28% (P ≤ 0.05). Rat liver ferritin (Sigma Chemical Co., St. Louis, Mo.) at 10⁻⁹ M, as reported previously (12, 17, 18), had no effect on colony formation by itself or in combination with M-CSF (data not shown) but completely abrogated colony formation by progenitors responding to LPS and the growth factor (P ≤ 0.05). H-ferritin was similarly inhibitory for the transitional progenitors (P ≤ 0.05); however, the ferroxidase-negative mutein had no effect on either one- or two-signal-dependent colony formation.

We have previously shown that antiferritin antibodies and antibodies specific for the 14-mer peptide Glu-Thr-Val-Ile-Met-Lys-Ala-Lys-Pro-Arg-Ala-Asn-Phe-Pro neutralize ferritin inhibition of in vitro myelopoiesis. While antiferritin inhibits stimulated production of the inhibitory monokine, antipeptide binds to and neutralizes the monokine (12). As shown in Table 1, inhibition by rat liver ferritin and H-ferritin was neutralized by both rabbit antiferritin immunoglobulin G (IgG) and affinity-purified rabbit antipeptide IgG (16). These data clearly suggested that H-ferritin, like rat liver ferritin, exerted its inhibitory effect through stimulated production of the previously described inhibitor (12). In light of the mutein’s failure to inhibit the two-signal progenitor response, the results further implicated a requirement for intrinsic ferroxidase activity in stimulated inhibitor production.

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P388D1 murine macrophage cells produce an antipeptide-neutralizable inhibitory monokine when stimulated with rat liver ferritin and M-CSF (12). Therefore, to directly assess the influence of intrinsic ferroxidase activity on monokine production, H-ferritin and the ferroxidase-negative mutein were tested for stimulation of inhibitor production by P388D1 cells. Rat liver ferritin, H-ferritin, or ferroxidase-negative mutein at 10^-9 M was added to the macrophage line cells (8 x 10^9/ml) in RPMI 1640 medium containing 3.3% endotoxin-free fetal bovine serum (HyClone Laboratories, Logan, Utah) and antibiotics and incubated with or without M-CSF (10^9 U/ml) for 8 h at 36°C in 7% CO_2. Serial dilutions of the 8-h medium samples were assayed for inhibitory activity in adherent-cell-depleted soft-agar assays (12). Prior depletion of adherent marrow cells diminishes the potential for inhibition by carryover ferritin in the supernatants, permitting detection of the inhibitor (12). Figure 2 shows the greatest sample dilutions yielding statistically significant inhibition (P ≤ 0.05) of two-signal-dependent colony formation (12). Supernatant media from unstimulated P388D1 cells and from P388D1 cells treated with M-CSF or the various ferritins alone were not inhibitory at dilutions of >1:10. Similarly, the supernatant medium from cells exposed to M-CSF and the mutein was not inhibitory, although that from P388D1 cells exposed to M-CSF and either rat liver ferritin or H-ferritin inhibited colony formation by the two-signal-dependent progenitors at equivalent dilutions of 1:160. Lastly, as shown in Table 2, the inhibitory effect of the M-CSF and H-ferritin supernatant medium was neutralized by antipeptide but not antiferritin IgG, as we have shown previ-

![Diagram](image-url)

FIG. 1. Schematic representation for positive and negative interactions of LPS and H-ferritin in regulation of M-CSF-dependent monocytopoiesis. Progenitors responsive solely to M-CSF represent macrophage lineage-committed macrophage CFU (CFU-M) derived from granulocyte-macrophage (GM)-CSF and interleukin 3 (IL-3)-responsive bipotential CFU-GM. Transition of CFU-GM to committed CFU-M expressing the M-CSF receptor C-fms progresses through an S-phase-positive (S^+) transitional progenitor stage, the pre-CFU-M. LPS stimulates transition of the pre-CFU-M to M-CSF responsiveness through a TNFα-dependent process that is sensitive to inhibition by H-ferritin. Evidence indicates that H-ferritin inhibition of pre-CFU-M stimulation is mediated by a cytokine produced by macrophages responding to both H-ferritin and M-CSF.

### TABLE 1. Influence of ferroxidase activity in ferritin inhibition of LPS-augmented progenitor responses to M-CSF and neutralization by antiferritin and antipeptide antibodies

<table>
<thead>
<tr>
<th>Costimulant and inhibitor treatment</th>
<th>No. of colonies (mean ± SE)</th>
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<tbody>
<tr>
<td></td>
<td>CSF</td>
</tr>
<tr>
<td>None (control)</td>
<td>123 ± 3</td>
</tr>
<tr>
<td>LPS</td>
<td>158 ± 6^a</td>
</tr>
<tr>
<td>LPS + rat liver ferritin</td>
<td>123 ± 5</td>
</tr>
<tr>
<td>LPS + H-ferritin</td>
<td>117 ± 3</td>
</tr>
<tr>
<td>LPS + ferroxidase-negative mutein</td>
<td>156 ± 5^a</td>
</tr>
</tbody>
</table>

^a LPS was used at 0.1 ng/ml; inhibitors were used at 10^-9 M.
^b Six-day colony response in cultures of 5 × 10^4 nucleated marrow cells stimulated with 750 U of M-CSF per ml, with or without costimulants and inhibitors. No colonies formed in cultures containing any of the additions without M-CSF.
^c Final concentration, 850 ng/ml.
^d Final concentration, 600 ng/ml.
^e P ≤ 0.05 (t test) compared with the respective control value.

### TABLE 2. Anti-14-mer peptide IgG neutralization of inhibitory supernatant activity produced by P388D1 cells treated with M-CSF and H-ferritin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of colonies (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF (control)</td>
<td>208 ± 9</td>
</tr>
<tr>
<td>CSF + LPS</td>
<td>297 ± 3^a</td>
</tr>
<tr>
<td>CSF + LPS + P388D1, supernatant</td>
<td>221 ± 6</td>
</tr>
<tr>
<td>CSF + LPS + P388D1, supernatant + antiferritin IgG</td>
<td>216 ± 3</td>
</tr>
<tr>
<td>CSF + LPS + P388D1, supernatant + antipeptide IgG</td>
<td>297 ± 8^c</td>
</tr>
</tbody>
</table>

^a Additions were as follows: LPS, 0.1 ng/ml; P388D1, supernatant, 1:80 dilution of supernatant medium from cells stimulated with M-CSF and 10^-9 M H-ferritin; antiferritin IgG, 850 ng/ml; antipeptide IgG, 600 ng/ml.
^b Six-day colony response in cultures of 7.5 × 10^4 adherent-cell-depleted nucleated marrow cells stimulated with 750 U of M-CSF per ml, with or without costimulant and inhibitors. No colonies formed in cultures containing any of the additions without M-CSF.
^c Significantly different from control value at P ≤ 0.05 (t test).
ules for rat liver ferritin (12). These results, as well as those shown in Table 1 and Fig. 2, are representative of at least three highly reproducible experiments with qualitatively similar results.

Although we have previously determined that predominantly light-chain ferritin is inactive in the two-signal-dependent culture system, we had not conclusively demonstrated that ferritins exclusively composed of heavy chains were active in this system. Previous investigation has revealed that the myelosuppressive activity of H-ferritin is restricted solely to the ferroxidase center of the heavy chain and is critically dependent upon ferritin reactivity with iron (4). The ferroxidase-negative mutein used assembles into stable ferritin protein with unaltered structure and cell binding properties (4). An earlier hypothesis to explain the influence of H-ferritin and lack of effect of this mutein on progenitors was the possibility of cellular iron deprivation through an intimate H-ferritin ferroxidase interference with transferrin transport of ferric iron and subsequent reduction of the transported iron to usable ferrous form (4). The present observations introduce the possibility that H-ferritin ferroxidase activity, whether by the proposed mechanism or a different one, influences production of a myelopoietic inhibitor by mature mononuclear phagocytes exposed to M-CSF (12). Production of this inhibitor requiring stimulation by both growth factor and H-ferritin is a unique process requiring further investigation for clarification.

Abnormalities have been observed in interactions between H-ferritin and hematopoietic cells during the progression of leukemias (1, 2), and it is reasonable to assume that production of the inhibitory monokine may significantly influence myelopoiesis and resistance to infection during these states. From an alternative viewpoint, it will be interesting to determine if the inhibitory monokine might have anti-inflammatory properties.

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REFERENCES


