

# Interleukin 1 Administration in Mice Produces Hypoferremia Despite Neutropenia

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## Abstract

To determine whether the hypoferremic response to inflammation requires neutrophils, we administered human recombinant IL-1 to mice made neutropenic with cyclophosphamide. With single intraperitoneal injections of IL-1 the plasma iron concentrations decreased significantly in mice with either normal neutrophil counts or neutropenia. After single injections transferrin concentrations were not significantly changed, but the decrease in serum iron lowered mean transferrin saturations from a baseline of 45 to 24–30% in nonneutropenic mice, and from 99 to 70–77% in neutropenic mice. Similar changes were observed after intraperitoneal injections of *Escherichia coli*. 4-d continuous infusions of IL-1 also led to reductions in serum iron concentrations, but transferrin concentrations doubled. The combination of a decrease in serum iron and an increase in transferrin concentration after chronic infusion in neutropenic mice led to a greater decline in mean transferrin saturations, from a baseline of 110 to 25%. In mice not given cyclophosphamide, chronic IL-1 infusion was associated with a reduction in mean hemoglobin concentrations from 14.7 to 13.5 g/dl, consistent with restricted availability of iron for erythropoiesis associated with low saturation of transferrin. We conclude that IL-1 can decrease the serum iron despite profound peripheral neutropenia and that transferrin is a positive acute phase reactant in the mouse.

## Introduction

The pathway whereby the monokine IL-1 mediates the induction of hypoferremia in inflammation (1, 2) is unknown, but at least two general mechanisms have been proposed. One hypothesis is that lactoferrin released from neutrophils is responsible for the rapid transport of iron from plasma to hepatocytes and macrophages (3, 4). The second is that hypoferremia is produced by an impaired outflow of iron from reticuloendothelial cells, hepatocytes, and intestinal epithelial cells resulting from an altered intracellular handling of iron (3, 5). Evidence for the lactoferrin mechanism includes the following observations: (a) lactoferrin is contained within the specific granules of neutrophils (6, 7) which seem to be the primary source of circulating lactoferrin (8, 9); (b) IL-1 leads to exocytosis of the contents of secondary granules with release of lactoferrin (10); (c) circulating lactoferrin may form lactoferrin-iron complexes (11) that are rapidly cleared from the plasma

by hepatocytes (12, 13), hepatic sinusoidal endothelial cells, Kupffer cells (14), and other macrophages (11, 15, 16); and (d) lactoferrin infusion produces hypoferremia in rats (11) and rabbits (4). Recently Goldblum and colleagues reported that induction of peripheral granulocytopenia in rats significantly reduced the percentage decline in serum iron between 0 and 6 h after IL-1 injection. They concluded that "granulocyte released lactoferrin is a specific carrier molecule for transport and removal of iron during the acute phase response" (4).

Clinical observations, however, document the occurrence of inflammatory hypoferremia despite peripheral neutropenia. Low circulating lactoferrin concentrations are found during neutropenia (8, 17, 18), and plasma lactoferrin is absent in agranulocytosis (19). Nonetheless, patients with neutropenia associated with nonmalignant conditions may have low plasma iron concentrations during inflammation (20), and patients undergoing bone marrow transplantation may substantially reduce the transferrin saturation during neutropenic febrile episodes (21). Leukemic patients with sepsis after intensive chemotherapy may develop hypoferremia despite neutropenia and low plasma lactoferrin concentrations (17).

To determine experimentally if circulating neutrophils are required for the hypoferremic response produced by IL-1 we examined the plasma iron after administration of human recombinant IL-1 to mice made neutropenic by treatment with cyclophosphamide.

## Methods

**IL-1.** Human recombinant IL-1 was obtained from Dr. Peter Lomedico of Hoffman-La Roche (Nutley, NJ). The purification and biological characterization of this protein have been reported (22). This protein was prepared from *Escherichia coli* and consisted of the carboxy terminal 154 amino acids of the 271 amino acids of the human IL-1 alpha precursor. Two lots of IL-1 were used in these investigations. (a) For analyzing the effect of an acute injection of IL-1 the preparation had a protein concentration of 60 µg/ml in 25 mM Trizma HCl, 0.8 M NaCl (pH 8.1), and a specific activity of  $3 \times 10^7$  lymphocyte activating factor (LAF)<sup>1</sup> units/mg (23). There was slight contamination with endotoxin, 800 limulus amebocyte lysate units/ml (24). For this study IL-1 was diluted in 0.1% sera-saline (prepared using pooled mouse sera) to final concentrations of 600 and 6,000 LAF units/ml. (b) For examining the effect of a continuous infusion of IL-1, the preparation had a protein concentration of 500 µg/ml in 30 mM Trizma HCl, 0.4 M NaCl (pH 7.8), and a specific activity of  $1 \times 10^9$  D10 units/mg (25) (1 LAF unit = 20–50 D10 units). Endotoxin content was minimal (~15 limulus amebocyte lysate units/ml). For infusion the IL-1 was diluted in 0.1% sera-saline and placed in 2,001 or 2,002 mini-osmotic pumps (Alza Corp., Palo Alto, CA) that deliver at constant rates of 1 or 0.5 µl/h.

**Cyclophosphamide.** Cyclophosphamide U. S. Pharmacopeia was obtained from Adria Laboratories (Columbus, OH) and diluted in normal saline to a concentration of 2,000 µg/ml and injected intraperitoneally at a dose of 250 µg/g body wt.

1. Abbreviations used in this paper: LAF, lymphocyte activating factor.

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**Trizma HCl.** Trizma HCl was obtained from Sigma Chemical Co. (St. Louis, MO) and diluted in 0.1% sera-saline for administration.

**E. coli.** Cultures of *E. coli*, A.T.C.C. No. 25922, were grown in tryptose phosphate broth overnight. The bacteria were washed three times in normal saline solution and resuspended in normal saline to a concentration of  $2 \times 10^7$  CFU/ml.

**Mice.** Male CF-1 mice weighing 25–35 g were obtained from Charles River Breeding Laboratories (Wilmington, MA). At the end of the study the animals were killed under light ether anesthesia by cardiac puncture and exsanguination. Blood was collected in heparinized syringes. White blood cell counts and hemoglobin concentrations were measured with a Coulter Counter ZF (Coulter Electronics, Inc., Hialeah, FL). Neutrophil counts were determined by differential counting of peripheral smears stained with Wright's stain and multiplication of the white blood cell count by the neutrophil fraction. Plasma iron concentrations and total iron binding capacities were determined using the methods of the International Committee for Standardization in Hematology (26, 27) modified for small quantities of plasma.

**Single injection of IL-1.** To determine the onset and duration of hypoferremia after single injections of IL-1, fasted normal mice were given intraperitoneal injections of 20 LAF units/g IL-1 or 1.2  $\mu$ g/ml Trizma HCl in 0.1% sera-saline. Cohorts of five animals from each treatment group were killed at 1, 2, 4, 6, 8, 10, and 12 h.

To examine the effect of IL-1 on neutropenic animals, cyclophosphamide or saline was administered intraperitoneally on days 1–3 to mice fed a low-iron diet obtained from ICN Nutritional Biochemicals (Cleveland, OH). The mice were fasted from the evening of day 3. On day 4 the mice were divided into four experimental groups with 10 mice in each group treated with cyclophosphamide and 10 treated with saline. At 0 h one group was injected intraperitoneally with 0.5 ml Trizma HCl, 0.26 mg/ml in 0.1% sera-saline; a second group was given 10 LAF units/g IL-1; a third group received 100 LAF units/g IL-1; and a fourth group received  $1 \times 10^7$  CFU *E. coli* in 0.5 ml sterile saline. The animals were killed at 8 h.

**IL-1 infusion in mice not given cyclophosphamide.** Osmotic pumps containing 0.16 mg/ml Trizma HCl in 0.1% sera-saline were implanted subcutaneously in five mice, and pumps containing IL-1 were implanted in six mice to provide 145 D10 units/g per h. The mice were killed after 94 h.

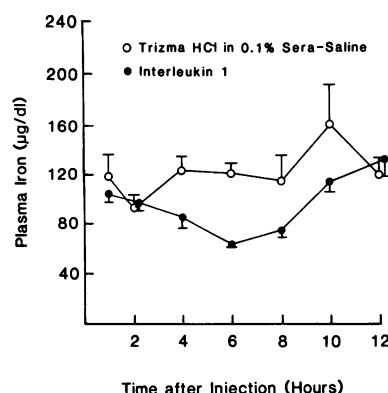
**IL-1 infusion in mice treated with cyclophosphamide.** Osmotic pumps containing 3.8  $\mu$ g/ml Trizma HCl in 0.1% sera-saline, or IL-1 to provide 14 D10 units/g per h, were implanted subcutaneously in nine mice. The concentration of IL-1 given to these animals was ~ one-tenth that given to mice not treated with cyclophosphamide. Cyclophosphamide was administered by subcutaneous injection daily for the first three days. The mice were killed on day 4, 99 h after the pumps were implanted.

**Statistics.** Independent sample *t* tests were used to compare mean values for neutrophil counts, plasma iron concentrations, total iron binding capacities, and transferrin saturations. For multiple comparisons the Bonferroni procedure was used to assure detection of an overall significance level of 0.05 (28, 29).

## Results

**Onset and duration of hypoferremia after acute injection of IL-1.** When compared with mice given Trizma HCl in 0.1% sera-saline, mice given IL-1 by acute injection experienced a significant reduction in mean plasma iron that began at 4 h and persisted through 8 h (Fig. 1). In subsequent studies we chose to examine the plasma iron concentration at 8 h.

**Acute injection of IL-1 in mice with and without cyclophosphamide treatment.** Compared with mice injected with saline, mice treated with cyclophosphamide for 3 d developed leukopenia, elevated plasma iron concentrations, decreased total



**Figure 1.** Effect of acute injection of 20 LAF units/g human recombinant IL-1 on plasma iron concentration in mice. Each point represents mean  $\pm$  SEM value for five mice, except  $n = 4$  at 1 h for mice given Trizma HCl in 0.1% sera-saline. Significance levels:  $P < 0.05$  at 4 h,  $P < 0.001$  at 6 h,  $P < 0.1$  at 8 h,  $P = \text{NS}$  for all other times.

iron binding capacities, and almost fully saturated transferrin (Table I).

When administered to mice treated with saline, IL-1 in concentrations of 10 and 100 LAF units/g was associated with significant increases in neutrophils and significant decreases in plasma iron concentrations and transferrin saturations compared with mice injected with Trizma HCl in 0.1% sera-saline. There were no significant changes in total iron binding capacities. The decrease in plasma iron with 100 LAF units/g IL-1 was significantly greater than that seen with 10 LAF units/g IL-1. Injection of  $1 \times 10^7$  CFU *E. coli* produced changes in white blood cells, plasma iron concentrations, and transferrin saturations similar to those seen with 100 LAF units/g IL-1, and a small increase in total iron binding capacity (Table I).

In mice that were neutropenic after cyclophosphamide treatment, IL-1 administration at both levels also resulted in significant decreases in plasma iron and transferrin saturations compared with injection of Trizma HCl in 0.1% sera-saline. There were also small but statistically significant increases in the neutrophil counts. Administration of  $1 \times 10^7$  CFU *E. coli* again produced changes in the parameters measured similar to the changes produced by 100 LAF units/g IL-1. Total iron binding capacities did not change significantly. 100 LAF units/g IL-1 was associated with a higher mean neutrophil count and a lower mean transferrin saturation than was seen with 10 LAF units/g, but there was no significant difference in the mean plasma iron concentrations.

The decrease in plasma iron was calculated for each of the animals treated with IL-1 and *E. coli* by subtracting the plasma iron concentration from the mean plasma iron concentration in the 8-h postinjection sample of the control group treated with Trizma HCl in 0.1% sera-saline. Mean values for decrease in plasma iron and mean absolute neutrophil counts are shown in Fig. 2. The mean decreases in plasma iron seen with administration of IL-1 and *E. coli* were significantly greater in the neutropenic animals when compared with animals without neutropenia ( $P < 0.001$ ).

**Continuous infusion of IL-1.** In untreated mice given an IL-1 infusion for 4 d (Table II), results for mean plasma iron and white blood cells after completion of infusion were comparable to those seen 8 h after a single injection of IL-1 (Table I), with a significant increase in neutrophils and decrease in iron when compared with animals given infusion of Trizma HCl in 0.1% sera-saline. In contrast to the observations after the acute injection of IL-1, the total iron binding capacity doubled. The decline in mean transferrin saturation to 25%

Table I. Acute Injection of IL-1 in Mice

Substance administered on day 4	<i>n</i>	Neutrophils	Serum iron	Total iron binding capacity	Transferrin saturation
		<i>10<sup>3</sup>/μl</i>	<i>μg/dl</i>	<i>μg/dl</i>	%
Mice treated with normal saline days 1–3					
Trizma HCl in 0.1% sera-saline	9	0.7±0.03	128±7	284±10	45±2
IL-1 (10 LAF units/g)	9	1.6±0.1*	106±7‡	317±10	30±3*
IL-1 (100 LAF units/g)	9	2.5±0.2*	75±4*	308±9	24±1*
<i>E. coli</i>	9	1.5±0.2*	88±7*	329±10‡	27±3*
Mice treated with cyclophosphamide days 1–3					
Trizma HCl in 0.1% sera-saline	9	0.2±0.1	234±10	236±11	99±2
IL-1 (10 LAF units/g)	10	0.5±0.03‡	162±7*	215±6	77±2*
IL-1 (100 LAF units/g)	7	0.3±0.04	157±3*	223±9	70±2*
<i>E. coli</i>	8	0.5±0.04	150±7*	216±5	69±3*

Represents day 4 values, mean  $\pm$  SEM. Significance level when compared with value for mice treated with Trizma HCl in 0.1% sera-saline: \* $P < 0.01$ ;  $^\ddagger P < 0.05$ .

with continuous infusion of IL-1 was greater than the decrease to 70 and 77% after acute injection of IL-1.

In a separate experiment, mice made neutropenic by cyclophosphamide treatment received IL-1 infusion for 99 h. These mice experienced a significant decrease in plasma iron and doubling of the total iron binding capacity when compared with animals that were given infusion of Trizma HCl in 0.1% sera-saline. There was no significant difference in white blood cell counts between the mice given IL-1 and those given Trizma HCl in 0.1% sera-saline.

In the mice not treated with cyclophosphamide, IL-1 infusion for 4 d significantly lowered hemoglobin concentration (Table II). The decrease in hemoglobin concentration in these mice was similar to that seen with cyclophosphamide treatment.

## Discussion

Human recombinant IL-1 administration by either acute injection or chronic infusion significantly decreased plasma iron concentrations in mice with either normal neutrophil counts

or severe neutropenia resulting from cyclophosphamide treatment. Similar findings were observed with injection of *E. coli*, possibly due to endogenous release of IL-1. Thus, our results suggest that factors other than release of lactoferrin by neutrophils may be involved in the hypoferremic effect of IL-1 and that further consideration should be given to impaired flow of iron from tissues to plasma as the explanation for hypoferrmia in inflammation.

Administration of IL-1 over 4 d by continuous infusion via subcutaneously implanted osmotic pumps permitted an approximation of physiologic conditions. In contrast to acute injection, the continuous infusion of IL-1 produced a marked rise in transferrin concentration as measured by total iron binding capacity (Table II). This increase in transferrin concentration in mice after prolonged IL-1 infusion contrasts with the decrease in transferrin concentration found in humans with chronic inflammation (30).

There are considerable interspecies differences in the details of iron metabolism and the acute phase response (31, 32). The plasma iron concentration in iron-replete mice is typically two or more times that in iron-replete humans. Mice transferrin concentrations are also higher (33). Transferrin is a negative acute phase reactant in man and a positive acute phase reactant in rabbits (32, 34). IL-1 stimulates cultured rabbit hepatocytes to synthesize transferrin (34). Our results suggest that chronic infusion of IL-1 stimulates transferrin synthesis in mice (Table II). In rabbits the serum transferrin concentration does not increase until 24 h after an inflammatory stimulus but then continues to increase for up to 72 h (34). Our observations in mice are consistent with these observations in rabbits. The transferrin concentration as measured by total iron binding capacity was not increased by 8 h after an acute injection of IL-1 (Table I) but the level doubled with 4 d of continuous IL-1 infusion (Table II). Thus, during inflammation humans and mice are similar in that both experience decreased plasma iron concentrations and transferrin saturations. They differ in that the transferrin concentration decreases in man but increases in mice. Because of interspecies differences in the metabolism of iron, caution is needed in extrapolating from these experimental results in mice to humans.

Cyclophosphamide-treated, neutropenic mice had a lower mean transferrin saturation (25%) after chronic infusion of

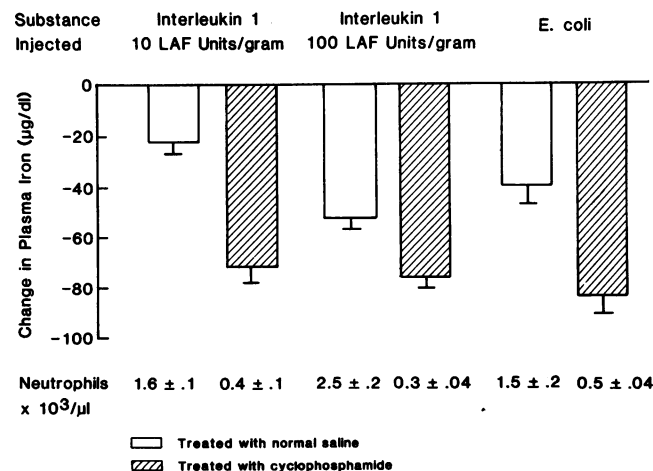


Figure 2. Mean  $\pm$  SEM decrease in plasma iron observed with acute injection of IL-1 and *E. coli* in neutropenic and normal mice. Significance levels:  $P < 0.001$  for each comparison.

Table II. 4-d Infusion of IL-1 in Mice

Substance infused by osmotic pumps	n	Neutrophils	Serum iron	Total iron binding capacity	Transferrin saturation	Hemoglobin
		$10^3/\mu\text{l}$	$\mu\text{g/dl}$	$\mu\text{g/dl}$	%	g/dl
Mice not given cyclophosphamide						
Trizma HCl in 0.1% sera-saline	5	$1.2 \pm 0.2$	$210 \pm 8$	$321 \pm 16$	$66 \pm 3$	$14.7 \pm 0.3$
IL-1 (145 D10 units/g per h)	6	$8.5 \pm 1.8$	$142 \pm 13$	$633 \pm 18$	$22 \pm 2$	$13.5 \pm 0.2$
P		<0.01	<0.001	<0.001	<0.001	<0.01
Mice treated with cyclophosphamide days 1-3						
Trizma HCl in 0.1% sera-saline	5	$0.1 \pm 0.04$	$342 \pm 53$	$318 \pm 21$	$110 \pm 21$	$13.6 \pm 0.4$
IL-1 (14 D10 units/g per h)	4	$0.1 \pm 0.1$	$172 \pm 15$	$700 \pm 30$	$25 \pm 2$	$13.5 \pm 0.5$
P		NS	<0.05	<0.001	<0.01	NS

Represents day 4 values, mean  $\pm$  SEM.

IL-1 than after a single injection (mean transferrin saturations 70–77%) because of the increase in transferrin concentration with chronic infusion. The magnitudes of the declines in serum iron concentrations were similar in both forms of administration (Tables I and II). Thus, with chronic IL-1 infusion in mice both the decline in serum iron and the increase in transferrin concentration contributed to a reduction in transferrin saturation to a level substantially below normal. The mechanisms responsible for the acute hypoferrremia observed after administration of IL-1 remain to be determined. With acute inflammation, a rapid decrease in the flow of iron from reticuloendothelial cells is observed that has been attributed to altered intracellular handling of iron (3). Konijn and Herskho have provided indirect evidence that inflammation provides a direct stimulus to apoferritin synthesis with subsequent diversion of iron away from the release pathway into ferritin stores (5), an action that could conceivably be mediated by IL-1. As yet, the effect of IL-1 on apoferritin synthesis has not been examined, and the role of apoferritin synthesis in the production of acute hypoferrremia awaits direct confirmation.

Iron uptake by tissues is directly related to transferrin saturation (35). Monoferric transferrin, which predominates with low transferrin saturations, delivers iron to the tissues less than one-fifth as efficiently as the diferric transferrin that is prevalent at high transferrin saturations (36). The low transferrin saturation with IL-1 infusion may have a physiologic role in restricting the availability of iron for erythropoiesis, as manifested by a significant reduction in hemoglobin concentration on day 4 in the mice not given cyclophosphamide (Table II). This finding resembles the change seen in the anemia of chronic disease in humans where iron release from reticuloendothelial cells is impaired and iron-deficient erythropoiesis results (30). Our observations are consistent with the hypothesis that IL-1 mediates the anemia of chronic disease (3). The decrease in hemoglobin concentration in mice given IL-1 was similar to that observed in mice with marrow suppression produced by cyclophosphamide (Table II).

The human recombinant IL-1 used in this study was highly purified (22) and it is unlikely that a hitherto unrecognized contaminant was responsible for the effects observed. The amounts of endotoxin present in the two preparations of IL-1 were less than concentrations that increase fibrinogen (37), produce leukocytosis (38), or decrease plasma iron (39). The validity of testing human recombinant IL-1 in mice is sup-

ported by previous studies that examined the effect of human recombinant IL-1 on plasma iron in mice and used the heat inactivated protein for control purposes (40). Although only circulating neutrophils were measured in our study, it is unlikely that lactoferrin derived from any remaining neutrophilic forms in the bone marrow and spleen could account for the observed hypoferrremia.

The conclusion of Goldblum and colleagues that the development of hypoferrremia after injection of IL-1 was blunted in rats made neutropenic by cyclophosphamide treatment (4) may differ from our findings because of the manner of data interpretation. Goldblum et al. expressed changes in serum iron as a percentage of baseline values. This method of analysis may not be optimal because of the effect that neutrophil depletion with cyclophosphamide would be expected to have on serum iron concentrations. A variety of cancer chemotherapy agents regularly produce a rise in the plasma iron concentrations (41) as observed in this study (Tables I and II). With an elevated basal plasma iron concentration in neutropenic animals, a smaller percentage decrease in plasma iron may represent an equivalent or larger absolute decrease in plasma iron when compared with control animals with a normal iron concentration. In our study the absolute declines in iron concentrations observed after acute injections of IL-1 and *E. coli* were greater in the neutropenic, hyperferrremic animals than in those with normal neutrophil counts (Fig. 2).

We have shown that single injections of human recombinant IL-1 in mice with normal circulating neutrophil counts or severe neutropenia produce significant reductions in plasma iron concentrations and transferrin saturations. Chronic administration of IL-1 leads to lowered plasma iron levels, a twofold rise in transferrin concentrations, and a further lowering of transferrin saturations. With chronic IL-1, hemoglobin concentrations decrease consistent with restricted availability of iron for erythropoiesis associated with low saturation of transferrin. We conclude that IL-1 can decrease serum iron despite profound peripheral neutropenia, and that transferrin is a positive acute phase reactant in the mouse.

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