Influence of Iron on In Vivo Proliferation and Lethality of L1210 Cells

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ABSTRACT The ability of iron to stimulate the growth of L1210 cells both in DBA-2 mice and in cell culture is evaluated. Although in vitro stimulation is absent, in vivo studies clearly indicate higher numbers of tumor cells in the presence of supplemental iron. When mice were given iron i.p., at levels comparable to clinical doses for humans (24 mg/kg body weight), the tumor load recovered from their peritoneum was substantially greater than from controls without iron supplements. Furthermore, at higher levels of supplemental iron (250 mg Fe/kg body weight), the pretreated animals inoculated with L1210 cells died in 9.7 d whereas controls died in 12.2 d (i.e., 25% faster). As expected, the lower iron dose (24 mg/kg) also resulted in shorter life spans, although the effects were less striking. It is the belief of these authors that these data support the opinion that "anemia of chronic disease" associated with leukemia and possibly other malignancies may represent a host defense mechanism as has been postulated by others (1, 8). J. Nutr. *115*: 369–374, 1985.

INDEXING KEY WORDS iron • neoplasm

A recent review by Weinberg has called for increased research into the role of iron metabolism in neoplasm (1). The review points out that disease states that induce iron overload have been frequently associated with an enhanced incidence of malignancy. This situation is probably best exemplified in individuals with ideopathic hemochromatosis, in which a major cause of death is cancer (2, 3). Another case, the excessive oral ingestion of iron, in the form of home-brewed spirits, in South African gold miners was associated with an extraordinarily high incidence of primary hepatoma (4–6). Furthermore, iron-dextran complex injections have been connected with the development of sarcoma at the injection site in animals and humans (7). Because of the deleterious effects of excess iron, it is not surprising that physiological systems would develop methods to withhold it.

It has been suggested that the anemia of chronic disease, which is often associated with malignancy, infection or inflammation, may represent a host defense mechanism whereby the body withholds iron from the "infectious" process (1, 8). In these situations total iron-binding capacity falls; ferritin-bound iron increases in the liver and spleen; and unsaturated serum ferritin and haptoglobin rise (1, 9, 10).

Although induction of neoplasm by iron has been supported by experimentation (11), there are no reports that document the potential of "supplemental" iron to facilitate the growth of neoplasm in vivo. It has on the other hand been shown that iron chelators such as hydroxamates, thiosemicarbazones, α-picolinic acid and 1,10-phenanthrolines block the transition from the G_1 to the S phase of mitosis in the Chinese hamster or in HeLa S3 cells (12–15). In our

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laboratories we have also shown that numerous catecholamide ligands are potent inhibitors of L1210 cell growth in vitro (16). This antineoplasic activity has now been shown to result from the inhibition of iron-dependent ribonucleotide reductase, a key enzyme in the biosynthesis of DNA (17). In this report we demonstrate that the administration of iron to DBA-2 mice alters the growth characteristics of L1210 cells in vivo.

MATERIALS AND METHODS

Cell line and animals. The L1210 cell line was obtained from Grace Cancer Drug Center, Roswell Park Memorial Institute, Buffalo, NY, compliments of C. W. Porter. This cell line was maintained in DBA-2 mice obtained from Frederick Cancer Research Center, Ft. Detrick, Frederick, MD. All in vivo experimentation utilized the same mice.

In vivo study design and analytical methods. L1210 cells were harvested from a DBA-2 mouse that had been inoculated with 1×10^6 viable cells 7–8 d earlier. Cells were removed from the animal's abdomen by two washings with 6 ml of cold sterile normal saline (Abbott Lifecare 100-ml infusion bags, Abbott Labs, North Chicago, IL). These cells were centrifuged at $300 \times g$ for 6 min. The supernatant was decanted and discarded, and the cells were resuspended in cold normal saline, centrifuged, decanted and resuspended. Cell concentration was determined, utilizing an appropriate dilution, followed by visual counting by using a hemocytometer (AO Spencer Instruments, Buffalo, NY) and compound microscope (AO Spencer). Cell viability was periodically determined through the use of a trypan blue exclusion technique (0.5% in unbuffered 0.9% NaCl solution).

In each study, 24 mice were injected i.p. with iron-dextran complex (Proferdex, 5% elemental iron–20% of 5 kilodaltons dextran Fisons Corp., Bedford, MA) at a dose equivalent to 12.5 mg/kg body weight of elemental iron every 12 h for two doses. This dose is similar to that recommended by the manufacturer (30 mg/kg) for total iron-replacement therapy in humans with a hemoglobin-iron concentration of 8 g/dl (18). An equal number of mice, which served as controls, were injected with iron-free dextran (Pfizons Ltd., Loughborough, U.K.) at a dose equivalent to that delivered as the iron-dextran complex (50 mg/kg every 12 h for two doses). Another 24 mice were injected i.p. with an iron-dextran complex dose of 125 mg/kg body weight every 12 h for two doses. Their controls received iron-free dextran at a dose of 500 mg/kg every 12 h for two doses.

Cells were reinoculated i.p. into iron-supplemented mice and into an equal number of control mice at 1×10^6 viable cells/mouse, within 1 h of harvesting, in cold normal saline in approximately a 0.25- to 0.35-ml volume. Inoculations were begun 6 h after the second injection of iron-dextran complex was delivered. Twenty-four hours after inoculation and daily thereafter, for 8 d, three mice each were randomly chosen from both the iron-supplemented and control groups and were killed. Cells were harvested, and the number of cells recovered from the abdomen per mouse was determined as previously described.

Death rate experiments were carried out for iron-treated and control groups with 35 mice pretreated with iron (doses of 12.5 mg/kg or 125 mg/kg for two doses) and inoculated with 1×10^5 L1210 cells along with 35 controls who received cells but no iron. Another 10 mice each were given iron (doses of 12.5 mg/kg or 125 mg/kg for two doses), but no L1210 cells, and were monitored for lethal toxicity of the iron supplements.

All animal experiments were run in duplicate.

Diet. All animals were fed a nonpurified diet (Rodent Lab Chow 5001, Ralston Purina Co., St. Louis, MO). According to the manufacturer, this formulation contains 0.2 mg elemental iron per gram of diet.

Serum iron assay. Total serum iron measurements were determined for animals receiving high or low doses of iron-dextran complex and high or low doses of dextran (controls). Iron concentration was quantified on protein-free serum by using a bathophenanthroline/sodium acetate colorimetric assay (19).

In vitro methods. Lastly, the effects of supplemental iron on in vitro growth were measured. L1210 cells were maintained in exponential growth between 5.0×10^4 and 1.0×10^6 cells/ml in RPMI 1630 tissue

culture medium (Gibco, Grand Island Biological Co., Grand Island, NY) containing 5% fetal calf's serum (Gibco) 0.062 mg/ml, penicillin G sodium (Sigma Chemical Co., St. Louis, MO) 0.135 mg/ml streptomycin sulfate (Sigma Chemical Co.). Culture medium was incubated at 37°C in ambient atmosphere in capped 20-ml sterile culture flasks (Corning #25100, Corning, NY). Iron was delivered to the medium from atomic absorption standard solution, 1.003 Fe g/ml in 2% HNO_3 (Aldrich Chemical Co., Milwaukee, WI) as a dilute solution in previously iron-free phosphate-buffered saline. The resulting dilute iron stock solution was adjusted to pH 7.0 with 10% NaOH (wt/vol). Iron was added to increase the iron concentration in the medium by 1.79×10^{-6} M to 1.79×10^{-4} M. Cell growth was quantitated by using electronic particle counting (Model ZF Coulter Counter, Coulter Electronics, Hialeah, FL) and confirmed periodically with hemocytometer estimates. Viability was assessed as previously described.

Statistical methods. Analysis of differences in the growth rate of i.p. L1210 cells in the presence versus absence of supplemental iron was carried out. Least squares regression analysis was used to indicate data from which study days best fit a log-linear model indicative of exponential cell proliferation. A test for heterogeneity of the slopes in the log-linear portion of the curves was used to determine whether the slopes for the iron-supplemented group's regression line differed significantly from that of the control group. This is an *F*-test of the additional reduction in the sum of the squares that occurred by allowing different regression coefficients for the two groups (20). In the mice receiving a given dose of iron-dextran complex both the total serum iron concentration and the death rate were compared to control animals by using a one-tailed *t*-test and Z-test, respectively, for one factor and two factor levels (21).

RESULTS

The effect of a relatively low iron supplement (24 mg/kg body weight) on the increase in neoplastic cell number of mice infected with L1210 cells is indicated in figure 1. On each day after inoculation, the

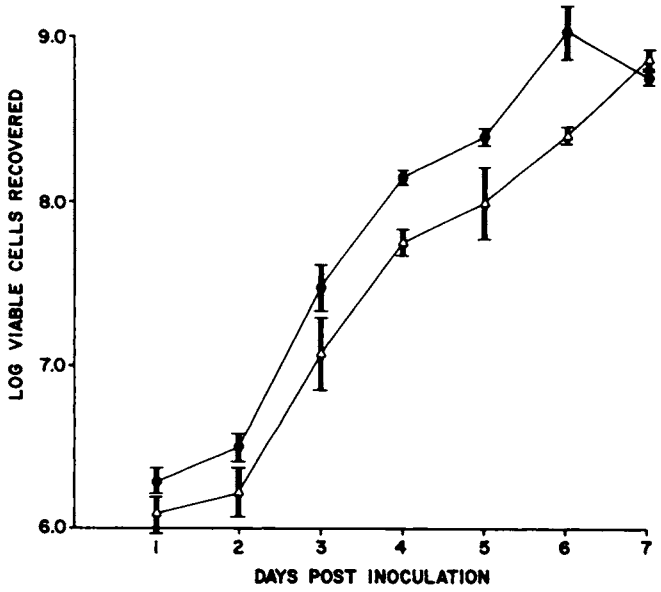

Fig. 1 Effect of iron-dextran complex (dose of 12.5 mg/kg body weight of elemental iron i.p. for two doses) on recoverable i.p. tumor load of DBA-2 mice inoculated with 1×10^6 L1210 cells. Slopes of the log-linear portion of the growth curves confirms rate of growth for the iron group (●) exceeded that of controls (△); $P = 0.02$ for the *F*-test. Graph represents a composite of duplicate experiments ($n = 48$ per factor level). Bar brackets depict day-to-day standard error for each mean value.

recoverable tumor load was larger in mice pretreated with the metal, a difference that increased daily from a low of 163% of controls for d 1 to 460% of controls on d 6. Statistical analysis of the log-linear portion of the growth curves, d 2–6 and 2–7 for the iron and control groups, respectively, indicates that the slopes differ significantly ($P < 0.031$) for the F-test. The effects of a higher dose iron supplement (250 mg/kg) are indicated in figure 2. Once again, on each day after inoculation the number of cells per tumor was appreciably higher in the iron-treated mice. Growth rates differed significantly ($P < 0.005$) on d 2–6 and 2–7 for the iron-treated and control groups, respectively.

Death rate analysis of the effect of high doses of iron produced a startling shortening of the life span for iron-treated mice (fig. 3). Mean life span for controls was 12.2 d post inoculation, while iron-treated mice lived only 9.7 d as a mean ($P < 0.005$ from a one-tailed Z-test). Low dose injections of iron were also associated with an alteration in survival time. A statistically significant earlier death in the presence of iron was still observed with a difference in survival time of 0.7 d for iron-treated versus control animals ($P < 0.005$ from a one-tailed Z-test). Acute lethal toxicity was not observed over a 20-d period in animals receiving iron dextran without L1210 cells at either iron dosage.

Finally, results of total serum iron measurements (table 1) indicate that only high doses of iron were associated with an increase in serum iron.

Growth rate of L1210 cells in culture medium was not accelerated in the presence of supplemental iron. Indeed as the concentration of added iron approached the solubility limits for the metal in this medium (1.79×10^{-4} M), growth rates were somewhat slower.

DISCUSSION

Let us, for a moment, assume that the drop in serum iron observed in the presence of malignancy represents an effort to deprive the neoplasm of accessible iron. It would then seem reasonable that the peritoneal administration of iron could increase

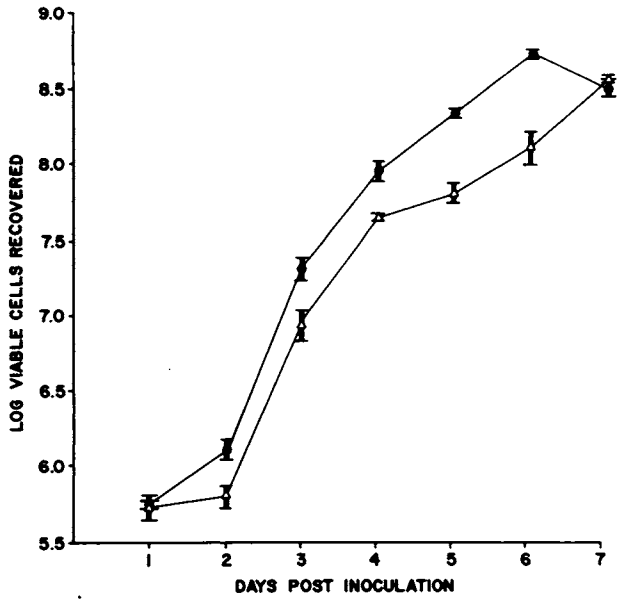

Fig. 2 Effects of iron-dextran complex (dose of 125 mg/kg body weight of elemental iron i.p. for two doses) on recoverable i.p. tumor load of DBA-2 mice inoculated with 1×10^6 L1210 cells. ●, Iron-dextran-treated animals; △, dextran-treated animals. $P = 0.03$ for the F-test. Graph represents a composite of duplicate experiments ($n = 48$ per factor level). Bar brackets indicate day-to-day standard error.

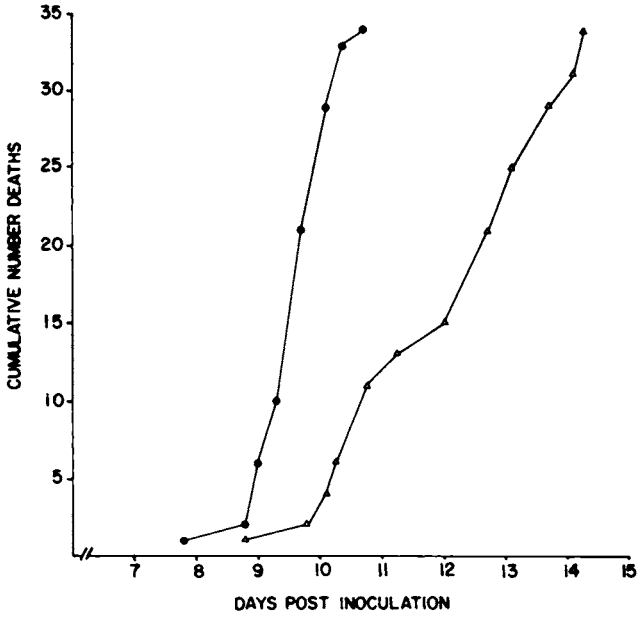

Fig. 3 Life-span alteration induced by iron-dextran complex (●) (125 mg/kg body weight of elemental iron i.p. for two doses) in DBA-2 mice or control DBA-2 mice (△) inoculated with 1×10^5 L1210 cells. $P < 0.005$ for one-tailed Z-test.

serum iron saturation and lead to the mitotic response observed in these experiments. Although a similar degree of growth enhancement of the i.p. tumor was observed in animals receiving either dose of iron-dextran complex, there appeared to be a difference in death rate. The larger iron supplement (250 mg/kg body weight) was associated with a more pronounced shortening in life span, and only this dose resulted in an increase in total serum iron. As iron-dextran is a slowly dissociating complex (18), the lower dose (24 mg/kg) may exert only a local i.p. growth enhancement. The higher dose (250 mg/kg), which was capable of enhancing systemic iron saturation, as shown by increased total serum iron concentration, may effect a systemic enhancement of neoplastic proliferation and/or metastasis. At the present time, this laboratory is studying the ability of iron to alter metastatic potential.

The association between anemia and malignancy is well established (22). Anemia is often the first observed sign of neoplasm, and 60% of all oncology patients are anemic. This anemia of chronic disease may be the result of autoimmune hemolysis; crowding of erythroid stem cells in the bone marrow; and/or increased storage of iron by tissue-bound ferritin of the reticuloendothelial system (1, 8, 23). It is this redistribution of iron into the reticuloendothelial system that may represent a form of host

TABLE 1

Total serum iron concentrations of DBA-2 mice receiving two i.p. injections of iron-dextran complex or dextran alone[1,2]

Injection			
	Dose		
Parenteral product	Dextran	Fe	Total serum iron
	mg/kg body wt		*mg/100 ml*
Dextran	500		221 ± 31.1*
Iron-dextran complex	500	125	368 ± 28.9*
Dextran	50		210 ± 21.9
Iron-dextran complex	50	12.5	203 ± 12.6

[1]Values are averages ± SD for four mice in the dextran-treated groups and five mice in the group treated with iron-dextran complex. Iron-free dextran molecular mass was 5–10 kilodaltons. [2]Significantly different from the other groups by one-tailed *t*-test: *$P < 0.0025$.

defense mechanism (1). By reducing availability of iron to the malignancy, the host might be attenuating neoplastic growth. Our observations reported herein would support this hypothesis.

Although the 24-mg dose of elemental iron (per kilogram body weight) is of the magnitude suggested as total replacement therapy for a moderate anemia in humans (whose hemoglobin-iron measures 8 g/dl), the 250-mg dose (per kilogram body weight) is significantly above the range for therapeutic dosage (18). In either case both of these doses represent a substantial supplement above normal dietary iron intake.

Further study into the role of iron metabolism in the pathogenicity of malignancy is warranted to more clearly define the clinical significance of these observations.

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