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Tissue distribution and clearance kinetics of non-transferrin-bound iron in the hypotransferrinemic mouse: A rodent model for hemochromatosis

(atransferrinemia/hemosiderosis/iron absorption)

C. M. CRAVEN*, J. ALEXANDER†, M. ELDRIDGE*, J. P. KUSHNER†, S. BERNSTEIN‡, AND J. KAPLAN*§

Departments of *Pathology and †Medicine, University of Utah College of Medicine, Salt Lake City, UT 84132; and ‡The Jackson Laboratories, Bar Harbor, ME 04609

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ABSTRACT **Genetically hypotransferrinemic mice accumulate iron in the liver and pancreas. A similar pattern of tissue iron accumulation occurs in humans with hereditary hemochromatosis. In both disorders, there is a decreased plasma concentration of apotransferrin. To test the hypothesis that nontransferrin-bound iron exists and is cleared by the parenchymal tissues, the tissue distribution of ^{59}Fe was studied in animals lacking apotransferrin. Two groups of animals were used: normal rats and mice whose transferrin had been saturated by an intravenous injection of nonradiolabeled iron, and mice with congenital hypotransferrinemia. In control animals, injected ^{59}Fe was found primarily in the bone marrow and spleen. In the transferrin iron-saturated animals, injected ^{59}Fe accumulated in the liver and pancreas. Gastrointestinally absorbed iron in hypotransferrinemic or transferrin iron-saturated mice was deposited in the liver. This indicates that newly absorbed iron is released from mucosal cells not bound to transferrin. Clearance studies demonstrated that transferrin-bound ^{59}Fe was removed from the circulation of rats with a half-time of 50 min. In transferrin iron-saturated animals, injected ^{59}Fe was removed with a half-time of <30 s. Analysis of the distribution of ^{59}Fe in serum samples by polyacrylamide gel electrophoresis demonstrated the presence of ^{59}Fe not bound to transferrin. These results demonstrate the existence of and an uptake system for non-transferrin-bound iron. These observations support the hypothesis that parenchymal iron overload is a consequence of reduced concentrations of apotransferrin.**

Iron is an essential nutrient for all mammalian cells and is generally found in plasma bound to transferrin (Tf). Tf-bound iron is transported to various sites of utilization and delivered to cells via Tf receptors. *In vitro* studies have shown that the amount of Tf-bound iron accumulated by cells is dependent on receptor number. Receptor number is regulated by the cell's metabolic needs, preventing the accumulation of excess cellular iron (1, 2).

Hereditary hemochromatosis is characterized by the triad of increased iron absorption by gastrointestinal cells, high or total iron saturation of plasma Tf, and abnormal iron deposition in parenchymal cells (3, 4). The mechanism of this excessive iron deposition is unknown. One hypothesis is that it may result from unregulated Tf receptor activity. Our studies, however, have shown that Tf receptor number and regulation are normal in lymphocytes and fibroblasts from individuals with hemochromatosis (5). The number and regulation of Tf receptors in the liver or pancreas is not well established (6–8).

A strain of hypotransferrinemic (HP) (BALB/cj-hpx/hpx) mice with an autosomal recessive condition closely resembling human congenital atransferrinemia has been identified (S.B., unpublished data; see refs. 9–11). These mice (HP) were found to have a hypochromic microcytic anemia and were growth-retarded at birth. Neonatal HP homozygotes die soon after birth but can be life-spared by weekly injections of whole mouse serum or Tf, even though the serum Tf levels in the life-spared animals rarely exceeded 1% of normal values. The life-spared adults develop parenchymal iron overload with massive iron deposition in the liver and pancreas. The parenchymal iron accumulation was not caused simply by the serum injections, as increased tissue iron was also found in the liver of fetal and neonatal HP mice.

We suggest that the similar pattern of tissue iron deposition in the two disparate disorders of hereditary hemochromatosis and hypotransferrinemia is due to a reduced concentration of plasma apotransferrin (apoTf). Without adequate apoTf, gastrointestinally absorbed iron accumulates in tissues by a mechanism that is independent of Tf. The cells that express this alternative pathway to the greatest extent are parenchymal cells, such as hepatocytes. In this communication, we present studies testing this hypothesis.

MATERIALS AND METHODS

Chemicals. $^{59}FeCl_3$ was purchased from New England Nuclear. Ferric citrate solution was obtained from Diagnostic Systems (Wilmington, DE). 3-(2-Pyridyl)-5,6-bis(4-phenylsulfonic acid) 1,2,4-triazine (Ferrozine) was purchased from Sigma. Mouse serum, purified mouse Tf, and anti-mouse Tf IgG were purchased from Cappel Laboratories (West Chester, PA). Rat Tf was isolated from serum by the method of Sawatzski (12). Kodak NTB-2 emulsion was purchased from Eastman.

Animals. Adult HP mice (BALB/cj-hpx/hpx), which had been life-spared by weekly injections of mouse serum, normal mice (BALB/cj-+/+), and Sprague–Dawley rats (200–220 g) were used for these studies. All animals were given standard rodent chow and water ad libitum.

Iron Saturation of Tf. Plasma Tf was transiently saturated by the intravenous injection of 0.1 ml of ferric citrate in 0.1 M citric acid buffer (pH 6.6) (70 μg for mice, 1.0 mg for rats). Controls were injected with an identical amount of citric acid buffer. Blood samples were obtained at determined time points. To calculate the Tf saturation, serum iron and total iron binding capacity were determined colorimetrically (13).

Gastrointestinal Iron Absorption. Iron absorption was measured in HP mice, normal mice, and mice that had been made anemic by serial retroorbital phlebotomies. The hematocrit and reticulocyte counts were determined in each group of animals (14). The mice were fasted overnight and given 0.1 ml

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Abbreviations: Tf, transferrin; HP, hypotransferrinemic; apoTf, apotransferrin.

§To whom reprint requests should be addressed.

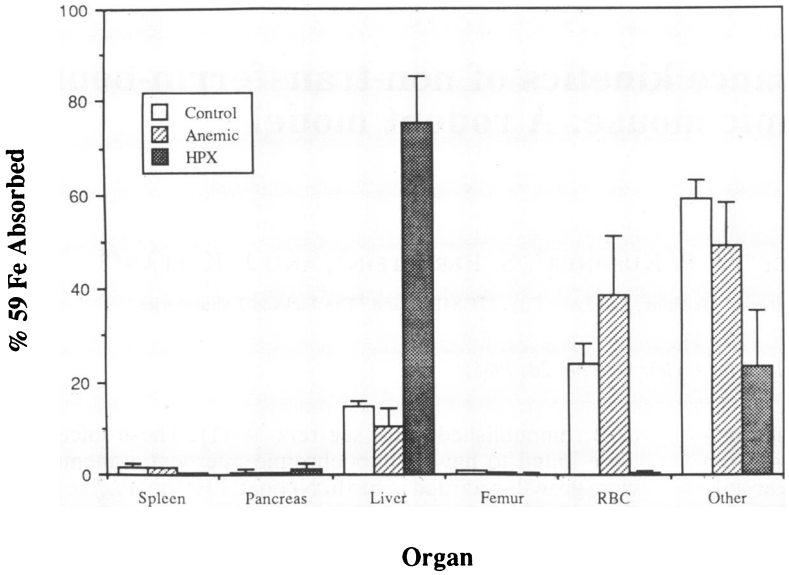

FIG. 1. Distribution of gastrointestinally absorbed ^{59}Fe in control, anemic, and HP mice. Normal mice were placed in groups ($n \geq 6$) and their baseline hematocrits and reticulocyte counts were determined. One group of mice was made anemic by daily phlebotomies. The normal, anemic, and HP mice were fasted overnight. Each group was given 0.1 ml of 0.4 nM ^{59}Fe in 1.0 M ascorbic acid in phosphate-buffered saline by gavage. Three days later, the mice were exsanguinated, and their organs were dissected. Hematocrit and reticulocyte counts were determined and the amount of radioactivity in specified organs was determined. The final hematocrits and the reticulocyte counts for each group were as follows: control, 58.0% (±4.1) and 4.2% (±1.3); anemic, 44.2% (±2.4) and 20.2% (±12.3); HP, 32.7% (±2.9) and 14.9% (±6.7). The data are presented as the mean percentage of the radioactivity found in each organ relative to the total counts recovered less those in the gastrointestinal tract. Error bars denote SEM. Tissues designated "Other" include the remaining viscera and carcass.

of ^{59}Fe in 1.0 M ascorbic acid in phosphate-buffered saline by gavage. At specified times, the mice were exsanguinated, and the amount of radioactivity in blood, whole organs, and carcass was determined using a Beckman Gamma 8000 Counting System. The amount of iron absorbed was defined as the amount of ^{59}Fe recovered in the entire animal excluding the gastrointestinal tract.

Iron Clearance and Distribution. ^{59}Fe (specific activity, 35.22 mCi/mg; 1 Ci = 37 GBq) in 0.1 ml of 0.1 M citric acid buffer (pH 6.6) was intravenously injected into mice (1 μCi) and rats (80 μCi). Mice were injected simultaneously with ^{59}Fe and nonradioactive iron. Rats received the nonradioactive iron intravenously 10 min prior to injection of ^{59}Fe. To determine clearance, blood samples were obtained at various times, and plasma radioactivity was measured. To determine the tissue distribution of ^{59}Fe after injection, animals were sacrificed at specified times and radioactivity was measured in whole mouse organs or weighed samples of rat tissues.

Gel Electrophoresis and Autoradiography. Aliquots of serum obtained from rats at specified times following the injection of ^{59}Fe were subjected to nondenaturing PAGE with a buffer system of 1.5 M Tris·HCl (pH 8.9) in the separating gel (7.5% acryl) and 0.5 M Tris·HCl (pH 6.7) in the stacking gel (3.0% acryl). Electrophoresis was for 8 hr at 25 mA. The gel was dried, and the position of the radiolabel was determined by autoradiography. As ^{59}Fe dissociates from Tf in acidic conditions, duplicate gels were stained with Coomassie blue to determine the positions of purified Tf. To determine the cellular location of ^{59}Fe, tissue autoradiography was done according to Ilford (15).

RESULTS

Cytochemical studies demonstrate that the parenchymal tissues of the HP mice accumulate iron. To determine whether newly absorbed iron accumulates in parenchymal tissues, the following experiment was performed. Groups of

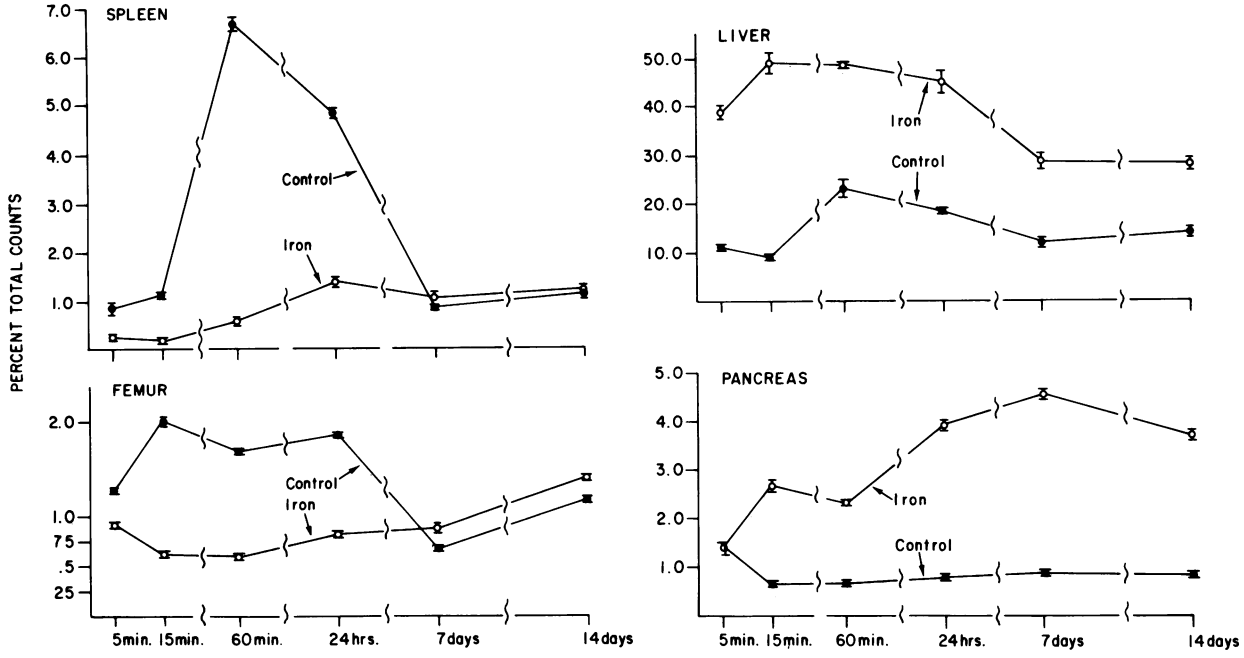

FIG. 2. Distribution of ^{59}Fe in organs from control and Tf iron-saturated mice. Groups of mice ($n \geq 6$) were injected with either ^{59}Fe or ^{59}Fe and 70 μg of ferric citrate. At specified times, mice were sacrificed, and the amount of radioactivity in selected organs was determined.

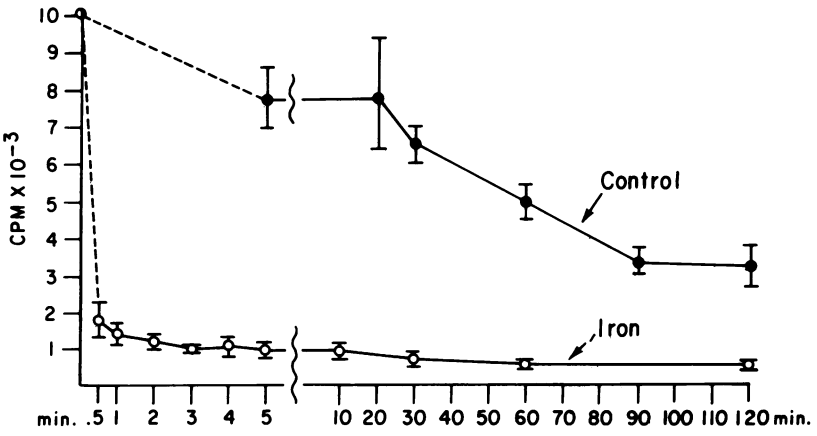

FIG. 3. Clearance of ^{59}Fe from the plasma of control and Tf iron-saturated rats. A group of rats were injected with nonradioactive iron. Ten minutes later, these rats as well as control animals were injected with ^{59}Fe. At specified times, samples of blood were removed and the amount of radioactivity was determined. ●, Radioactivity in normal animals; ○, radioactivity in Tf iron-saturated animals. Each point indicates mean ± SEM.

normal mice, HP mice with <1% of normal Tf levels, and mice made anemic by frequent bleeding were given a solution of ^{59}Fe dissolved in 1.0 M ascorbic acid by gavage. After 3 days, the animals were sacrificed, organs were dissected, and the amount of radioactivity absorbed and the organ distribution of radioactivity were determined. The percentage of iron absorbed by the anemic and HP mice were similar (22.5% ± 9.9% and 19.7% ± 3.8%) and greater than that of the control group (6.6% ± 2.2%). There was, however, a dramatic difference in the organ distribution of ^{59}Fe (Fig. 1). In the anemic mice, a significant fraction of the newly absorbed iron was found in erythrocytes, and a minor amount was found in the liver. In the HP mice, <1% of the ^{59}Fe was found in erythrocytes, while nearly all of the radioactivity was present in the liver. These data indicate that in animals with genetically low levels of Tf, newly absorbed iron is deposited into parenchymal tissues.

If the similarity in tissue iron distribution between HP mice and humans with hereditary hemochromatosis is the result of a decreased concentration of apoTf, then normal animals with highly saturated Tf should display an altered distribution of tissue iron, with increased iron delivery to the liver. We tested this hypothesis by intravenously injecting normal animals with sufficient ferric citrate to saturate the total iron-binding capacity of plasma Tf and determining the tissue distribution of injected ^{59}Fe. The iron saturation of Tf was confirmed by measuring serum iron and total iron-binding capacity. The dosage of intravenously injected nonradiolabeled iron was sufficient to transiently exceed the normal iron binding capacity by 2-fold in both rats and mice. In control mice, the highest concentration of radioactive iron was found in the spleen and bone marrow (Fig. 2). The organ distribution of ^{59}Fe in the Tf iron-saturated animals was markedly different. These animals had increased amounts of ^{59}Fe in the liver and pancreas and decreased amounts in the bone marrow and spleen. In mice, this abnormal distribution of ^{59}Fe persisted 14 days after the initial injection of the ^{59}Fe.

We also performed similar experiments in rats. The larger blood volume of the rat allowed us to do serial phlebotomies in a single animal and measure clearance of ^{59}Fe. In control animals, the rate of disappearance of a tracer dose of intravenously injected ^{59}Fe fit first-order kinetics (Fig. 3). The half-time for clearance was 50 min, which agrees well with published values for Tf-bound iron (16). To confirm that the ^{59}Fe in the control animals was bound to Tf, samples of plasma were analyzed by PAGE. All of the radiolabeled iron in the plasma of control animals comigrated with purified rat Tf (Fig. 4).

Radically different clearance kinetics were observed when a similar dose of ^{59}Fe was injected into rats whose Tf was saturated with nonradioactive iron. In these animals, >80% of the injected radioactivity was cleared from the plasma by 30 s. The distribution of radioactivity in serum analyzed by PAGE was also different. Two radioactive bands were seen: one that comigrated with rat Tf and another that migrated more rapidly toward the cathode. As the clearance of radioactivity in the experimental group was very rapid, it was necessary to apply more of the sample to the gels to achieve an equivalent amount of radioactivity per lane, as in the control group. The trace amount of apoTf that remained unsaturated in the plasma also bound ^{59}Fe and was more prominent with the greater amount of serum applied. Based on the distribution of radiolabel and the measured clearance of Tf-bound ^{59}Fe, we calculated that >90% of the initial dose was not bound to Tf. A similar rapid clearance of serum ^{59}Fe was observed in mice whose Tf was transiently iron-saturated.

Saturation of Tf with nonradioactive iron resulted in an alteration in the organ distribution of radioactivity in the rat (Fig. 5). At 3 hr after injection of $^{59}FeCl_3$, rats with iron-saturated Tf were found to have a 6-fold increase in specific activity (cpm per g wet wt of tissue) of ^{59}Fe in the liver, and a 9-fold increase in the pancreas. As was found in the Tf iron-saturated mouse, splenic and bone marrow (sternal) radioactivity was significantly reduced compared to controls, being lower by factors of 8 and 2.5, respectively. Tissue autoradiography confirmed that the ^{59}Fe was in parenchymal cells of the liver and the pancreas.

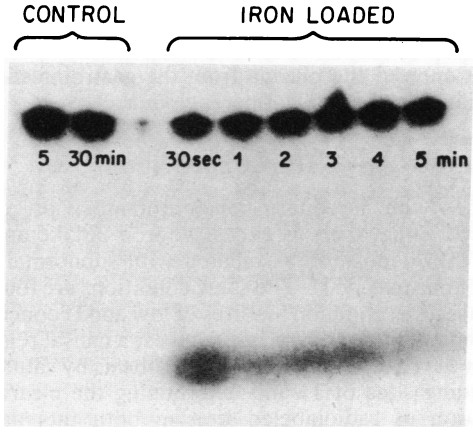

FIG. 4. Examination of the distribution of ^{59}Fe in plasma by PAGE and autoradiography. Selected serum samples from the experiment described in Fig. 3 were analyzed by PAGE. The position of the ^{59}Fe was determined by autoradiography. The position of purified rat Tf was determined on duplicate gels stained with Coomassie blue. To obtain similar amounts of radioactivity in each well, a 10 times greater amount of the serum from the Tf iron-saturated rat was applied.

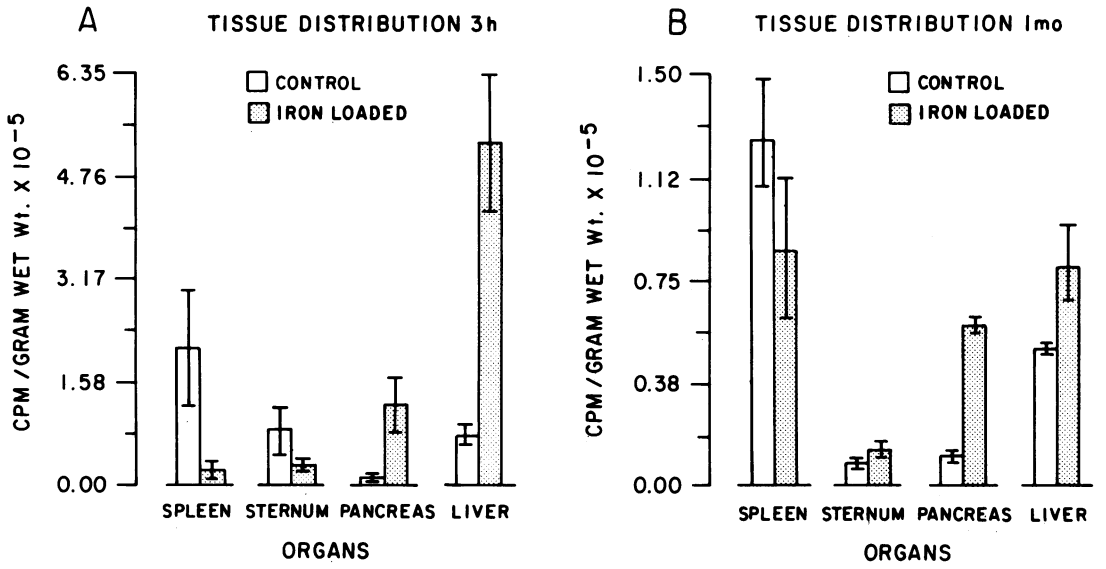

FIG. 5. Tissue distribution of ^{59}Fe in control and Tf iron-saturated rats. Animals were treated as described in Fig. 3, except that at specified times the rats were sacrificed, selected organs were removed and weighed, and radioactivity was determined. The data in this experiment are expressed as the mean specific activity (cpm per g wet wt of tissue) ± SEM.

To determine whether the abnormal iron deposition observed in the transiently Tf iron-saturated rats was persistent, the distribution of ^{59}Fe was examined 30 days after injection of ^{59}Fe (Fig. 5). The specific activity of the radiolabel remained elevated in the liver and pancreas when compared to controls. To ensure that a significant portion of injected ^{59}Fe was not excreted in the urine or feces, we measured the total radioactivity in all the organs tested and were able to account for 90% of the initial radioactivity at either 3 hr or 30 days.

These studies demonstrated that ^{59}Fe injected into the plasma of Tf iron-saturated animals was rapidly cleared by parenchymal tissues. To determine whether the organ distribution of ^{59}Fe absorbed by the gastrointestinal tract was affected by Tf iron saturation, we performed the following experiment. Normal mice or mice whose Tf had been saturated by the intravenous injection of nonradiolabeled Fe were given ^{59}Fe gavage. One hour later, the organ distribution of ^{59}Fe was determined. The amount absorbed by the two groups was similar, but the organ distribution was dramatically different (Fig. 6). More than 70% of the newly absorbed iron in the Tf iron-saturated group was deposited into the liver. Thus, in normal mice without apoTf, as in the HP mice, iron that entered the plasma from the gastrointestinal tract accumulated in the hepatic parenchyma.

DISCUSSION

In humans with hereditary hemochromatosis or congenital atransferrinemia, there is excessive iron uptake and parenchymal iron deposition associated with subnormal concentration of plasma apoTf. The same conditions are found in the HP mouse. The common features of low apoTf concentration and parenchymal iron overload suggest a causal relationship between the two. We tested this hypothesis by saturating the iron-binding sites of Tf and determining the clearance and distribution of radiolabeled iron in both rats and mice. Injection of ^{59}Fe into Tf iron-saturated rats resulted in a rapid disappearance of the radiolabel from plasma (Fig. 2). Analysis by PAGE indicated the presence of ^{59}Fe in a form that was not associated with Tf.

The organ distribution and plasma clearance of ^{59}Fe was altered when the concentration of apoTf was decreased by saturating the Tf with nonradioactive iron. Non-Tf-bound iron was cleared with a half-life of <30 s, compared to 50 min for Tf-bound iron. Both injected and absorbed ^{59}Fe was found in the parenchymal cells of the liver. Even though the saturation of Tf was transient, the abnormal tissue iron distribution was persistent. These results demonstrate the existence of an iron uptake system that is not mediated by Tf.

Similar observations have been previously reported by others. Laurell, in 1947, described a more rapid clearance of iron from plasma when the iron-binding capacity of Tf was exceeded (17). Others have confirmed this observation in both humans and experimental animals (18, 19). For example, Hershko and associates described an iron species not bound to Tf in the serum of thalassemic patients (20, 21). The early experiments were done before the mechanism by which Tf-bound iron enters erythrocytes had been defined (22). The

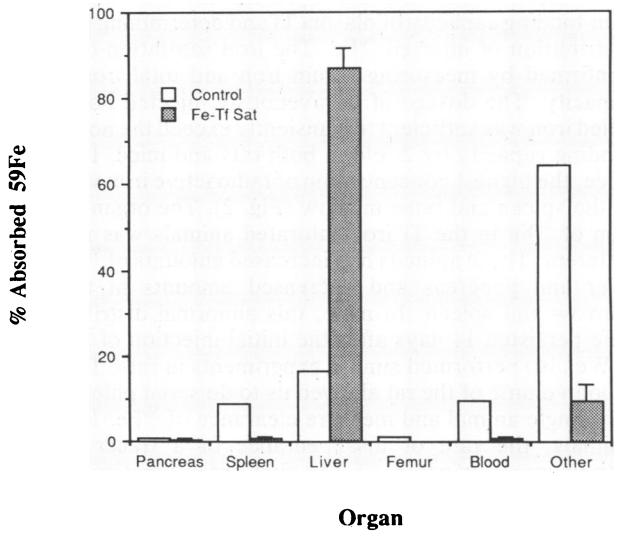

FIG. 6. Distribution of gastrointestinally absorbed iron in Tf iron-saturated mice and control mice. The Tf iron-saturated group was injected with 70 μg of iron in citric acid buffer; the control group was injected with buffer alone. Ten minutes later, both groups were given ^{59}Fe by gavage as described. The animals were sacrificed after a further 60 min. The gastrointestinal tract was excised, and the amount of radioactivity in the remaining organs was determined. The data are presented as described in Fig. 1.

rapid deposition of non-Tf-bound iron into hepatocytes and other parenchymal cells has not been well recognized or investigated. Brissot and co-workers, however, have recently demonstrated an uptake system for ionic iron in isolated and perfused livers (23, 24).

In this communication, we demonstrated that not only does the liver acquire non-Tf-bound iron, but that the pancreas also cleared intravenously injected non-Tf-bound iron. This suggests that the pancreas possesses the uptake system for non-Tf-bound iron and may explain how individuals with hereditary hemochromatosis, or the HP mice, acquire excess pancreatic iron. Most of the radiolabeled iron taken up by the liver and pancreas remained in these tissues over the time course of these experiments, even though the iron saturation of Tf was transient. This observation suggests that parenchymal iron is not released unless there is an increase in erythropoietic demand, such as is seen in hereditary hemochromatosis patients following phlebotomy. In HP mice, even though there is an increase in erythropoietic demand, as evidenced by an increased reticulocyte count, without Tf, iron remains inappropriately sequestered.

Previous studies indicated that iron absorption continues from the gastrointestinal tract when Tf is saturated (19, 25, 27). These studies, coupled with the observation that humans with atransferrinemia and the HP mouse are iron-loaded, suggest that apoTf is not an obligate carrier in mucosal iron release. Our studies have shown that the HP mouse, in fact, absorbs more ^{59}Fe than normal. ApoTf is necessary, however, to bind and transport newly absorbed iron to the normal site of utilization: the erythroid marrow. Without normal concentrations of apoTf, parenchymal iron accumulation might exceed Tf-mediated iron delivery to the marrow, resulting in tissue iron overload.

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