Iron Absorption in Hypotransferrinemic Mice

By Saundra S. Buys, Carol B. Martin, Marlies Eldridge, James P. Kushner, and Jerry Kaplan

We used a unique animal model, the hypotransferrinemic (Htx) mouse, to examine the role of transferrin (Tf) in gastrointestinal iron uptake. Despite the absence of Tf, Htx animals hyperabsorb iron. Transfusion of red blood cells sufficient to normalize the hematocrit and reticulocyte count resulted in a return of iron absorption to normal values. These studies indicate that Tf does not play an obligate role in iron absorption, either as a carrier or as a humoral signal regulating absorption. Transfer of plasma or whole blood from Htx mice or from other animal models of iron hyperabsorption to normal mice did not cause an increase in iron absorption in recipient animals. Using the plasma or blood transfer approach, we have been unable to detect a humoral regulator of gastrointestinal iron absorption.

The mechanism and regulation of gastrointestinal iron absorption has been investigated for decades. Gastrointestinal iron absorption is responsive to erythropoietic activity, and increased erythropoiesis (particularly ineffective erythropoiesis) results in increased iron absorption. Numerous studies have been performed in an attempt to detect a humoral factor that regulates iron absorption. Most studies have used plasma or tissue extracts from hypoxic animals, iron-depleted animals, or animals with hemolytic anemia. Plasma or extracts have been transferred into normal recipients, and iron absorption measured. No convincing evidence of a humoral regulator has been reported.1,2

The failure to find a plasma factor that regulates iron absorption has resulted in the development of the plasma iron turnover (PIT) hypothesis.3 This hypothesis states that the regulation of iron absorption is related to iron use (ie, that the rate of loss of iron from transferrin [Tf] defines the rate of iron absorption). Increased erythropoiesis would result in increased iron demand and a faster turnover of Tf iron. This hypothesis accounted for two observations: that iron absorption is generally correlated with increased iron demand, and that iron absorption is independent of the concentration of Tf in plasma or the degree to which Tf is saturated with iron. A problem in testing this theory is that it has not been possible to manipulate the turnover of iron from Tf without first altering the rate of erythropoiesis.

In this study we have used a unique animal model, the hypotransferrinemic (Htx) mouse, to explore the role of plasma Tf in iron absorption. The Htx phenotype is the result of a splicing defect in the Tf gene, resulting in an unstable Tf message and extremely low concentrations of circulating Tf.4 Animals homozygous for the defect have plasma Tf concentrations that are less than 1% of normal littermates, and are anemic but iron overloaded.5 Affected animals die soon after birth, but can be life-spared by the administration of either mouse or human serum (or Tf). These animals were used to examine the role of Tf in the regulation of iron absorption. Our studies indicate that bone marrow activity can regulate iron absorption even in the absence of Tf.

Materials and Methods

Animals. Animals were housed in the University of Utah Institutional Animal Care and Use Committee (IACUC)-approved facility. Htx mice were identified and maintained as previously described.6 Htx animals received intraperitoneal injections of normal mouse serum or purified mouse Tf each week. Some animals received 0.3 mL of a 6 mg/mL solution of human Tf intraperitoneally each week. On this protocol animals maintained a hematocrit (Hct) of 27% to 35%. Even after prolonged exposure to human Tf, no antihuman Tf antibodies could be detected in these animals (unpublished observation). Blood was obtained by retro-orbital puncture using a heparinized capillary tube. Intravenous injections were administered via lateral tail vein. Gastric lavage was performed using a blunt-tipped needle.6

Iron absorption. Measurement of iron absorption was performed as reported previously.6 Briefly, 55Fe as FeC13 was diluted in 0.1 mL 1 mol/L ascorbate in phosphate-buffered saline containing 6 mg unlabeled elemental Fe (as FeC13). The solution was administered intragastrically using a blunt-tipped gavage needle to animals that had been fasting for 2 to 3 hours. Animals were then fasted overnight in wire-bottom cages to prevent coprophagia and were killed by CO2 inhalation the following morning. In some experiments, animals were killed 4 hours after gavage. The 4-hour absorption data were identical to the 24-hour data. The gastrointestinal (GI) tract was removed and the radioactivity in the remaining organs and carcass was determined using a Beckman Gamma Counter (Beckman, Irvine, CA). Percent absorption was defined as the amount of radioactivity in the whole animal minus the GI tract, divided by the amount instilled by gavage. Results are shown as the mean ± SEM. The significance of differences between experimental groups was determined by Student's t-test.

Transfusion of Htx mice. Htx animals received intraperitoneal injections of red blood cells (RBCs) obtained from normal animals to correct anemia and suppress erythropoiesis. Cells were washed sufficiently to remove plasma Tf bound to cells. Animals received approximately 0.25 mL RBCs daily until the Hct increased into the normal range. The Hct was kept in the normal range for 5 to 7 days before iron absorption studies were performed. Intraperitoneal Tf was withheld during this time.

Plasma transfer experiments. Plasma or whole blood for intravenous or intraperitoneal injection into recipient animals was obtained from three sources: normal controls, anemic mice or rats, and Htx mice. Anemia in otherwise normal mice was produced by phenylhydrazine. Phenylhydrazine solutions were prepared by dissolving acetyl-phenylhydrazine (Sigma, St Louis, MO) in 37°C saline and flushing to maintain a hematocrit of 20% to 35%. Animals were anesthetized with pentobarbital and the superior vena cava cannulated to allow collection of blood. Blood was obtained as a bolus dose and transferred into a 50 mL syringe containing 10% ethylenediamine tetra-acetic acid. This solution was then divided into 2 mL aliquots and stored at -20°C until use. Plasma transfer experiments were performed as follows: animals were anesthetized with pentobarbital and blood or plasma obtained by retro-orbital puncture was administered intravenously or intraperitoneally.
0.9% sodium chloride to make a 2 mg/mL solution. The solution was then filtered and 0.5 mL of the solution was injected intraperitoneally each day for 3 consecutive days, or on days 1, 3, and 7. Blood was obtained on day 6 of the first regimen or day 10 of the second. Plasma from rats that had been rendered anemic using a combination of thiophenol and bleeding was used in some experiments. Blood from mice that had been repeatedly phlebotomized was used in other experiments.

Blood for transfer experiments was obtained by exsanguinating mice via retro-orbital puncture using heparinized capillary tubes, and by exsanguinating rats via cardiac puncture. If plasma was to be transferred, whole blood was centrifuged in microcapillary tubes. Blood or plasma was stored on ice and pooled for intravenous or intraperitoneal injection into recipient animals. The time from starting to bleed donors to injecting blood products into recipients varied depending on the number of animals used, but was generally 1 to 4 hours.

RESULTS

Effect of transfusion on iron absorption. As previously shown, the rate of iron absorption in Htx mice exceeds that of control animals (Table 1). The effect of normalization of the Hct and RBC production on iron absorption was assessed after RBC transfusion. Animals received washed packed RBCs by intraperitoneal injection until the Hct was well within the normal range, which generally took three injections of 0.25 mL packed cells. Control Htx animals received an equal volume of intraperitoneal saline. Iron absorption was measured after the animals had maintained a normal Hct for 5 to 7 days. To document that the transfused RBCs contained no Tf that could have confounded interpretation of the experiment, Tf concentration in plasma from transfused mice and in the supernatant from the last RBC wash was assayed by enzyme-linked immunosorbent assay (ELISA). The ELISA found no detectable Tf in the supernatant fluid, and a concentration of Tf in the plasma of transfused animals of 0.77 ± 0.15 mg/dL, a value similar to that of untransfused Htx mice (1.05 ± 0.26 mg/dL; normal animals, 314 ± 60 mg/dL). Increasing the Hct to normal values reduced erythropoiesis to normal values as assayed by reticulocyte production and resulted in a decrease in iron absorption into the same range as that seen in control animals (Table 1). These results show that Htx animals do not require Tf for iron absorption, either as an iron carrier in the gut mucosal cell or as a signal for iron uptake.

Search for a transferable humoral substance controlling iron absorption. To provide additional evidence for or against a humoral substance that is responsible for regulating iron absorption, plasma or whole blood from animals that had been shown to hyperabsorb iron was administered intravenously or intraperitoneally to control animals, and the effect on iron absorption was determined. Sources of blood included Htx mice, mice that had received phenylhydrazine to induce hemolysis, mice that had previously been bled, and rats made anemic with a combination of thiophenol and bleeding. Several different doses of plasma and schedules of iron instillation into the gut were used to try to detect an increase in iron absorption. In none of our experiments could we conclusively show an increase in iron absorption after transfer of plasma or whole blood from animals that hyperabsorb iron to control animals. Similarly, injection of high-reticulocyte whole blood into recipients, which has been shown to increase PIT, had no effect on iron absorption (6.0% ± 2.4% absorption in controls; 5.9% ± 2.5% absorption in reticulocyte-rich blood recipients; \( P = .97 \)).

DISCUSSION

Because of the well-defined role for Tf in iron transport and cellular delivery, it has been assumed that Tf plays an important mechanistic role in iron absorption. Two observations counter this assumption. First, individuals with totally saturated Tf, such as those with hereditary hemochromatosis, have no apotransferrin (apoTf) available for binding iron, yet they hyperabsorb iron. Experimentally manipulated humans or animals with totally saturated Tf also continue to absorb iron. Second, there has been a rare genetic condition in both humans and mice in which Tf concentrations are extremely reduced. Affected individuals (or animals) do not have decreased iron absorption and, in fact, hyperabsorb iron. We have taken advantage of the mouse mutant termed Htx, which is a genetic model of the human disease atransferrinemia, to investigate the role of Tf in iron absorption. As in the human disease, these mice absorb iron excessively. Because these animals are anemic, the observation that they hyperabsorb iron is not surprising. The PIT theory, however, which relates iron absorption to rate of use of Tf iron, would not necessarily predict that animals with extremely low Tf concentrations would hyperabsorb iron. The effect of Tf itself on iron absorption in Htx animals is difficult to assess, because administration of Tf sufficient to increase the Hct into the normal range increases RBC production, which would be expected to increase iron absorption. The effect on iron absorption produced by correction of the anemia in Htx mice was therefore assessed by transfusing RBCs from

### Table 1. Effect of RBC Transfusion on Iron Absorption in Htx Mice

<table>
<thead>
<tr>
<th>Experiment</th>
<th>% Absorption</th>
<th>Hct (%)</th>
<th>Reticulocyte Count (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong> (7)</td>
<td>4.5 ± 0.6</td>
<td>52.9 ± 3.2</td>
<td>2.1 ± 1.8</td>
</tr>
<tr>
<td>Htx (4)</td>
<td>42.1 ± 22.9</td>
<td>38.2 ± 0.8</td>
<td>16.0 ± 6.0</td>
</tr>
<tr>
<td>Transfused Htx (5)</td>
<td>9.1 ± 7.6</td>
<td>72.4 ± 6.5</td>
<td>2.3 ± 2.9</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (8)</td>
<td>6.0 ± 2.1</td>
<td>63.9 ± 5.2</td>
<td>3.4 ± 1.5</td>
</tr>
<tr>
<td>Htx (6)</td>
<td>54.4 ± 24.0</td>
<td>39.7 ± 3.7</td>
<td>52.8 ± 28.9</td>
</tr>
<tr>
<td>Transfused Htx (6)</td>
<td>13.0 ± 6.8</td>
<td>65.3 ± 9.3</td>
<td>5.8 ± 5.1</td>
</tr>
</tbody>
</table>

Transfused Htx mice received intraperitoneal injections of washed packed RBCs from normal mice as described in Materials and Methods. Control mice (normal BALB/C) and Htx mice received intraperitoneal normal saline. Iron absorption, Hct, and reticulocyte counts were then assayed. Numbers in parentheses refer to the number of animals in each group. \( P \) values: experiment 1: control and Htx \( P = .03 \); control and transfused Htx \( P = .28 \); experiment 2: control and Htx \( P = .004 \); control and transfused Htx \( P = .11 \).

*Details of the plasma transfer experiments will be provided upon request.*
normal littermates. Because it appears that there is only one Tf gene, Tf is absent not only from the plasma but from the Gl mucosa. This finding excludes an obligate role for Tf in iron absorption.

It has been suggested that Tf may provide an important signal for iron absorption. The PIT theory states that the rate of use of iron, specifically the rate of loss of iron from Tf molecules, directly governs the rate of iron absorption.

Cavill et al. proposed a modification of the PIT model that incorporated iron stores: iron absorption = intestinal iron stores/(total body iron stores × PIT). This theory has been hard to test because of the difficulty in regulating Tf turnover independently of hematopoiesis. Under normal conditions, any manipulation that increases erythropoiesis also increases Tf-mediated iron delivery. The Htx model allowed for regulation of hematopoiesis without affecting Tf concentration, and, thus, for examination of the relationship between Tf, hematopoiesis, and iron uptake. The studies presented here in the Htx mouse show that Tf is not an obligate sensor of iron turnover and provide evidence against the PIT hypothesis.

We have indirectly confirmed this observation by a different approach. Mice were injected with reticulocyte-rich blood, a situation that should lead to increased Tf iron use. We observed no increase in GI iron uptake, a result similar to that of Raja et al. These observations further suggest that an increase in Tf iron use is not causally linked to iron absorption.

Our studies also addressed the possibility of a humoral substance providing the signal that mediates increased iron absorption. Studies have purported to show a transferable substance that increases iron absorption. In some studies, the data do not support the conclusions of the authors, and in others the effect of erythropoietin was not taken into account.

Erythropoietin has no direct role in mediating or signaling iron absorption, but it does increase erythropoiesis, which in turn increases iron absorption. The majority of these studies, including ours, have not detected a transferable substance. There are several reasons why a humoral factor may not be detected in the type of assay we used. The factor may be inactivated during handling, or may need to be present at a specific and crucial time before or during iron absorption. We have attempted to avoid these potential difficulties by rapid collection and transfer of donor plasma, and by varying the schedule of administration. We used plasma from animals that absorb iron most avidly, the Htx mice, and we administered quantities of plasma equal to at least 50% of the plasma volume of the recipient mouse in an attempt to overcome the problem of subthreshold doses of the hypothetical agent. The failure to find a transferable substance that mediates iron absorption does not prove that it does not exist, but using a variety of sources and schedules of administration, we could find no evidence for a humoral substance that is capable of increasing iron absorption when administered passively to recipient animals.

This study shows that transfusion of the Htx mice with sufficient RBCs to restore the Hct and rate of RBC production to normal results in a decrease in iron absorption to control values. This observation shows that Tf is not mechanistically important in iron absorption, and provides evidence against the PIT hypothesis.

ACKNOWLEDGMENT

The authors thank Dr. Nancy Noble for help in obtaining the thiampenicol-treated rat plasma, and Cleo Ricks and Dorothy Wiscombe for assistance in preparation of the manuscript.

REFERENCES

Iron absorption in hypotransferrinemic mice [see comments]

SS Buys, CB Martin, M Eldridge, JP Kushner and J Kaplan

Updated information and services can be found at:
http://www.bloodjournal.org/content/78/12/3288.full.html

Articles on similar topics can be found in the following Blood collections