RED CELLS

Importance of Anemia and Transferrin Levels in the Regulation of Intestinal Iron Absorption in Hypotransferrinemic Mice

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The hypotransferrinemic mouse (trf<sup>hpx</sup>) is a mutant strain exhibiting transferrin deficiency, marked anemia, hyperabsorption of iron, and elevated hepatic iron stores. We set out to investigate the relative roles of anemia and of transferrin in the malregulation of intestinal iron absorption in these animals. Transfusion of erythrocytes obtained from littermate controls increased hemoglobin levels and reduced reticulocyte counts in recipient animals. Although mucosal to carcass<sup>59</sup>Fe transfer was reduced, total duodenal iron uptake was not significantly affected. Iron absorption in homozygotes, in contrast to littermate controls, was not reduced by hyperoxia. Mouse transferrin injections, in the short term, increased delivery of iron to the marrow and raised hemoglobin levels. Although mucosal transfer and total iron uptake were reduced at the higher transferrin doses, total uptake was still higher than in controls. Daily injections of mouse/human transferrin for 3 weeks from weaning, normalized hemoglobin values, and markedly reduced liver iron and intestinal iron absorption values in trf<sup>hpx</sup> mice. When such daily-injected mice were left for a week to allow transferrin clearance, iron absorption values were significantly enhanced; hemoglobin or hepatic iron levels were, however, not significantly altered. These data indicate that hyperabsorption of iron in trf<sup>hpx</sup> mice is not solely because of the anemia; transferrin levels per se do affect iron absorption, possibly via a direct effect on the intestinal mucosa.

MATERIALS AND METHODS

Reagents. All chemicals and biochemicals were from either Sigma Chemical Co Ltd (Poole, Dorset, UK) or BDH Chemicals (Poole). Radio-iron (<sup>59</sup>FeCl<sub>3</sub>) was obtained from NEN-Du Pont (specific activity 0.19 to 2.78TBq/g; Stevenage, Herts, UK).

Animals. The hypotransferrinemic mice originated from BALB/cJ background. Homozygous mice (trf<sup>hpx</sup>), which are phenotypically distinguishable at birth by their pale appearance, were maintained by weekly injections of mouse serum (150 µg to 1 mg transferrin) as described previously<sup>9</sup> Heterozygotes were differentiated from the wild-type controls by the reduction in serum transferrin levels. Experiments/manipulations on trf<sup>hpx</sup> mice were performed a week after the last serum maintenance dose, thus allowing for almost complete clearance of exogenously administered transferrin.<sup>11</sup> Mice were 6 to 8 weeks of age at the time of study.

Transfusion of erythrocytes. Control (ie, mixture of heterozygotes and wild types) or trf<sup>hpx</sup> animals were injected intraperitoneally (i.p.) with either 0.15 mol/L NaCl (saline) or washed, packed erythrocytes (~250 µL) obtained from homozygotes, heterozygotes or wild-type mice, and studied 3 days post-single or -double (3-day interval between injections) transfusion. Washing of erythrocytes with saline 3 times was found to be adequate for removal of any plasma-associated transferrin. In some experiments, animals were given an injection of erythrocytes daily for 3 days and studied 2 to 5 days later. Preliminary experiments performed in animals that had been injected intravenously (IV) with cells/saline showed no differences compared with the i.p.-injected mice; the 2 groups have therefore been combined for analysis.

Transferrin injections. Homozygous mice were injected i.p. with either a single or double dose of commercially obtained mouse
transferrin (Chemicon Int, Temecula, CA), and studied 4 hours to 1 week later. In addition, some trf<sup>hpx</sup> mice were injected daily, starting from weaning, with a progressively increasing dose (1 to 3 mg) of human or mouse transferrin for 3 weeks; this injection regime was followed to account for mouse growth. Iron absorption studies were performed 6 to 18 hours or 1 week after the last injection. Mice injected on a daily basis for 3 weeks with human albumin received the usual weekly maintenance dose of transferrin (as serum).

**Transferrin determination.** Serum levels were assayed by a radial immunodiffusion technique<sup>14</sup> using 1% agar containing 0.02 mol/L sodium barbitone buffer (pH, 8.6) and appropriate amounts of either goat anti-human transferrin (Dynatech Labs, Billinghurst, Sussex, UK) or sheep anti-mouse transferrin (Chemicon Int). Purified human transferrin (Sigma) or mouse transferrin (Chemicon Int) was appropriately diluted and assayed at the same time to establish a calibration curve. The human and mouse antibodies did not cross-react with mouse or human transferrin, respectively.

**Tissue oxygen levels.** Duodenal pO<sub>2</sub> levels were determined polarographically in anesthetized mice by gently inserting a previously calibrated, thin, flexible wire-type electrode into the duodenal mucosa through a slit made distal to the point where the bile duct joins the small intestine. The body temperature was maintained by placing mice on a heated pad. Recordings on a Model pO-100 monitor (composed of an annunciator with built-in power supply; Inter-Medical Ltd, Nagoya, Japan) were not commenced until the electrode was well positioned and secured. The measurement of pO<sub>2</sub> in mammalian tissue via polarographic electrodes and other means is described in the review by Vanderkoor et al.<sup>13</sup>

**Iron absorption.** In situ tied-off duodenal segments were used to determine intestinal iron absorption.<sup>3,11</sup> A tracer dose of <sup>59</sup>Fe (as a ferric chelate of nitrilotriacetate, 1:2, in physiological medium) was injected intraluminally into the tied-off segment of the anesthetized animal. The duodenal intraluminal contents were gently flushed-out with warm saline before the injection of radioiron solution. All experiments were performed under V<sub>max</sub> conditions [Fe(III) = 250 µmol/L] and for an incubation period of 10 minutes. The segment was thereafter removed, flushed with ice-cold saline, weighed, and counted in a gamma counter (LKB Wallac Model 80000; Turku, Finland). Blood (cardiac puncture) and any other required tissues were also removed, weighed, and similarly counted. The carcass was counted in a well-type counter. Aliquots (10 µL) of the radioiron solution, acting as standard, were counted in both counters to normalize the <sup>59</sup>Fe counts. The activity of <sup>59</sup>Fe in the duodenal segment is referred to as “mucosal retention,” whereas the activity in the carcass reflects the “mucosal transfer.” The sum of the 2 parameters represents the “total mucosal uptake.” The activity of <sup>59</sup>Fe associated with the blood was calculated assuming a stable nonabsorbed ecf marker,<sup>20</sup> showed no difference between the control (8.7 ± 4.8 [3] pmol/mg/10 min) and trf<sup>hpx</sup> (8.7 ± 4.6 [3] pmol/mg/10 min), thus indicating that the enhanced absorption of iron is specific and not attributable to increased intestinal permeability.

**RESULTS**

Figure 1 shows the body weights and hemoglobin levels, and Table 1 shows the iron absorption parameters in control and homozygous hypotransferrinemic mice. The heterozygote and wild-type mice have been combined into a single control group because previous investigations<sup>3</sup> have shown no significant differences in iron absorption between the 2 groups.

Homozygous mice had stunted growth as reflected by the reduced body weights and were markedly anemic with a high degree of reticulocytosis (23.2 ± 5.5 [6%]) as compared with the control group (1.3 ± 1.0[6%]). Intestinal iron absorption studies showed a marked enhancement in trf<sup>hpx</sup> mice mainly because of increased transfer of radioiron from the mucosa to the plasma. Permeability studies performed with <sup>51</sup>Cr-EDTA, a stable nonabsorbed ecf marker,<sup>20</sup> showed no difference between the control (8.7 ± 4.8 [3] pmol/mg/10 min) and trf<sup>hpx</sup> (8.7 ± 4.6 [3] pmol/mg/10 min), thus indicating that the enhanced absorption of iron is specific and not attributable to increased intestinal permeability. Most of the absorbed radioiron (after a 10-minute incubation in a tied-off duodenal segment) in trf<sup>hpx</sup> mice was found in the liver (72 ± 8.1 [6%]), with little being incorporated into erythrocytes (0.63 ± 0.64 [6%]). In contrast, values for the liver and erythrocyte <sup>59</sup>Fe incorporation in the control group were 16.3 ± 8.0 (10%) and 11.9 ± 12.6 (10%), respectively. These values resemble those reported in control/mutant mice studied at 24 hours<sup>4</sup> and 3 days<sup>5</sup> postadministration of <sup>59</sup>FeCl<sub>3</sub> in phosphate buffer via the IV and oral route, respectively. At the time of our study, transferrin could not be detected immunologically in the serum of trf<sup>hpx</sup> mice (ie, <.05 mg/mL).

Transfusion of trf<sup>hpx</sup> animals on 2 occasions (with a 3-day gap in between injections), with either saline or erythrocytes obtained from homozygous animals, did not cause any significant changes in the hemoglobin, mucosal transfer, or total uptake of <sup>59</sup>Fe in recipient mice. It is noteworthy that injection of trf<sup>hpx</sup> erythrocytes was performed as an additional control for the transfusion experiments and shows that injection of red blood cells per se does not affect iron absorption. In contrast, a single or double transfusion of erythrocytes from control animals resulted in significant changes in the hemoglobin level in recipient trf<sup>hpx</sup> mice, but neither treatment resulted in a decrease in the total mucosal uptake of iron. The doubly transfused group, though exhibiting a significant decrease in the percent mucosal transfer values, showed a significant increase
in the mucosal retention of radioiron (Table 1); thus, no apparent change in overall uptake was evident. Reticulocyte counts decreased after the single- (15.1 ± 13.2 [3]%)[4] or double-erythrocyte transfusion (3.6 ± 3.5[4]% of control erythrocytes as compared with the saline-infused group (33.6 ± 7.4 [3]%).

Multiple transfusion of erythrocytes (3 times) induced a further small increase in the hemoglobin level; the values posttransfusion were comparable with the values in the control group. As the reduction in percent mucosal transfer was more prominent and the mucosal retention values were unaltered in the multiple-transfused group, the total absorption values showed a reduction, but the values failed to reach statistical significance ($P < .2$). It is noteworthy that the total uptake values were still significantly higher than those in the control group. Clearance of radioiron by the liver was marginally increased (82.5 ± 9.0 [6]%), whereas that by the erythrocytes was reduced (0.10 ± 0.24 [6%]) following the multiple transfusions. Mice studied 2 to 3 days after multiple transfusions also showed a similar, statistically insignificant reduction in total mucosal iron uptake (69.8 ± 17.4 [3] pmol/mg/10 min).

Liver and duodenal mucosal non-heme iron levels were unperturbed in the transfused groups compared with untreated trf$hpx$ mice (Fig 2). When the hepatic data were corrected for liver weights, a small but progressive increase in total iron level was observed with increasing transfusion of control erythrocytes. Splenic iron levels were also moderately increased following the triple-transfusion (1.56 ± 0.61 [6] nmol/mg vs 1.04 ± 0.71 [6]). When allowance was made for the spleen weights, the total iron content values were comparable with those seen in the noninjected group (362 ± 133 [6] nmol vs 365 ± 263 [6]). Control animals multiply transfused with erythrocytes from heterozygote/wild-type littermates had elevated liver weights and hepatic iron content (2.4 ± 0.5 [5] pmol v 1.9 ± 0.8 [10]) and exhibited small, insignificant, reductions in mucosal transfer (7.7 ± 1.6 [5] v 10.1 ± 5.4 [10] pmol/mg/10 min), percent mucosal transfer (27.6 ± 4.4 [5]% v 30 ± 10.5 [10]%), and total mucosal uptake (27.8 ± 4.6 [5] v 32.3 ± 12.6 [10] pmol/mg/10 min).

Effect of hyperoxia on iron absorption. Trf$hpx$ mice exposed to 40% O$_2$ for 3 days failed to show any marked changes in the degree of reticulocytosis (18%, 21%, n = 2) or in intestinal iron absorption (total uptake = 108 ± 33 [4] pmol/mg/10 min). Exposure for shorter periods (ie, 24 and 48 hours) was also without effect on iron absorption (109 and 168 pmol/mg/10 min, respectively). A statistically significant reduction in iron absorption was, however, evident in control mice similarly exposed to 3 days hyperoxia (20.0 ± 6.1 [6] v 32.3 ± 12.6 [10] pmol/mg/10 min, $P < .05$), owing to a reduction in both the mucosal retention (13.4 ± 4.0 [6] v 22.2 ± 8.1 [10] pmol/mg/10 min) and mucosal transfer (6.6 ± 3.6 [6] pmol/mg/10 min v 10.1 ± 5.4 [10]) of $^{59}$Fe.

Tissue oxygen levels. No difference was evident in the duodenal pO$_2$ levels between the trf$hpx$ mice (21 mm Hg, n = 2) and the control group (20.5 ± 1.8 [3] mm Hg). When both groups were, however, made to inhale 10% O$_2$ for 2 minutes via a small mouth piece and the measurements performed, a decrease of between 2 and 5 mm Hg was evident as compared with basal values. Conversely, inhalation of 40% O$_2$ resulted in an increase of 2 to 8 mm Hg. The mucosal oxygen levels determined with the thin wire electrode are within the range of values observed in mammalian small intestine by surface and microelectrode measurements.$^{21-23}$

Effect of transferrin injection(s) on intestinal iron absorption. To monitor the effects of transferrin, trf$hpx$ mice were studied at specific times after injection with various doses of mouse transferrin (Fig 3).

Circulating transferrin levels in trf$hpx$ mice were at the time of study dependent on the dose given and the period postinjection (Fig 3A). Increased incorporation of absorbed radioiron by immature red blood cells was apparent 4 to 5 hours after transferrin injection, and peaked at about 24 hours; thereafter, the red blood cell $^{59}$Fe incorporation values decreased and by 7
days the values had reverted to basal levels (Fig 3B). Alterations in the incorporation of iron into erythrocytes (and thus in hemoglobin values) were more striking with the larger transferrin doses. The increased $^{59}$Fe incorporation by erythrocytes 24 hours postinjection with 4.4 mg transferrin was associated with a significant decrease in radioiron clearance by the liver. Thereafter, as the red blood cell incorporation of $^{59}$Fe decreased, the liver clearance values equalled or even exceeded values seen in untreated trf$^{hyp}$ mice. The injected group, however, still exhibited enhanced intestinal iron absorption (Table 2). Homozygous mice given 8.8 mg (2 x 4.4 mg) of transferrin however, showed an appreciable reduction in total uptake, mainly because of reduced transfer of $^{59}$Fe from the mucosa to the portal circulation (Table 2). The uptake values were still higher ($P < .04$) than those in wild-type/heterozygotes (total mucosal uptake, 32.3 ± 12.6 [10] pmol/mg/10 min). It is noteworthy that at the time of experiment there were no significant alterations in either clearance of radioiron by the liver or total liver iron content (23.4 ± 6.3 [4] µmol v 21.2 ± 3.5 [6]) after dosing with 8.8 mg of transferrin. The fact that circulating transferrin levels 3 days postinjection with either the single or double dose (with a 3-day gap in between injections) of 4.4 mg transferrin are similar, indicates that transferrin clearance is rapid (half-life < 24 hours), and is in agreement with previous observations. 13

Daily injections of commercially available mouse transferrin (1 to 3 mg) over a 3-week period, starting from weaning, normalized hemoglobin levels, markedly reduced hepatic iron stores, and significantly decreased iron absorption in trf$^{hyp}$ mice.
(Tables 3 and 4). The iron absorption values were, however, still higher than the control group. Mice treated similarly for 3 weeks with mouse transferrin and then left for a week (without injections) so that circulating transferrin levels decreased to undetectable/negligible levels, showed a marked increase in both the mucosal transfer and total uptake ($P < .02$) without any change in liver iron content or hemoglobin values. The total uptake values were similar to those seen in normally maintained trf$hpx$ mice. Animals treated with human transferrin on a daily basis for 3 weeks exhibited a similar increase in the hemoglobin level and also showed normalized percent mucosal $59$Fe transfer and total mucosal uptake values. Liver iron stores were also reduced, though not to the same extent as in the mouse transferrin treated group. As before, mice left untreated for a week after 3 weeks of daily injections had undetectable circulating transferrin (ie, $<.05$ mg/mL) and yielded a significant increase in both mucosal transfer and total uptake, even though hemoglobin levels were normal. Circulating transferrin levels at the end of the 3-week period of daily injections were similar in both groups, suggesting that the protein, though from different sources, is cleared at similar rates.

Homozygous mice injected in a similar fashion for 3 weeks, but with human albumin, exhibited marked anemia, elevated hepatic iron stores, and enhanced mucosal retention, transfer, and total uptake values; these characteristics are similar to those seen in mice receiving maintenance serum injections only, and suggests that the effects of human transferrin on iron absorption are not because of possible induction of an immunologic reaction.

**DISCUSSION**

The trf$hpx$ mouse, a genetic strain with a virtual absence of circulating transferrin, shows similarities to the human conditions hemochromatosis and atransferrinemia. It is thus a useful animal model for investigating not only the regulation of iron absorption but also the mechanism of iron toxicity.$^{24}$

In spite of the surplus iron, the mutant strain exhibits a marked anemia of an iron-deficient nature; the sustained hyperabsorption of iron, though not surprising, is, however, intriguing because (1) animals lack transferrin, which is necessary for the delivery of iron as ‘iron-transferrin,’ the primary physiological source of iron for erythroid cells, and (2) changes in absorption are more marked than seen in other models with chronic anemia (ie, $\beta$-thalassemia,$^{11}$ thalassemia-deficient$^{12}$) or with enhanced reticuloctysis (phenylhydrazine-treated$^{25}$).

In this study we have investigated the relative importance of

| Table 2. Effect of Transferrin (Tf) Injections on Intestinal Iron Absorption |
|-----------------------------|---------------------|---------------------|---------------------|
| TF Dose (mg)                | n                   | Mucosal Retention   | Mucosal Transfer   |
|                             |                      | (pmol/mg/10 min)    | (pmol/mg/10 min)   |
| Nil                         | 6                   | 28.4 ± 10.7         | 63.2 ± 14.4        |
| 1                           | 6                   | 39.3 ± 14.5         | 67.2 ± 12.0        |
| 1                           | 4                   | 29.9 ± 12.9         | 57.3 ± 21.2        |
| 2 × 1 over 24 h             | 5                   | 47.2 ± 17.8         | 62.7 ± 12.1        |
| 2.2                         | 3                   | 33.6 ± 19.0         | 80.8 ± 33.2        |
| 4.4                         | 4                   | 30.0 ± 6.1          | 51.4 ± 13.0        |
| 4.4                         | 4                   | 23.2 ± 18.6         | 53.6 ± 38.9        |
| 2 × 4.4 over 1 wk           | 4                   | 33.0 ± 10.0         | 42.9 ± 27.7        |

Data: mean ± SD for (n) experiments.
Abbreviations: Δt, sampling time after the single or second injection. $^*$
$^†P < .02$, $^‡P < .006$ as compared with untreated homozygous mice.

| Table 3. Effect of Daily Transferrin Injections on Circulating Transferrin, Hemoglobin, and Liver Iron in Homozygous Hypotransferrinemic Mice |
|-----------------------------|---------------------|---------------------|---------------------|
| Group                      | Serum Transferrin (mg/mL) | Hemoglobin (g/100 mL) | Liver Iron (nmol/mg) |
|                            | Mouse               | Human               | Mouse               |
| Controls                   | 10                  | 2.1 ± 0.6$^*$         | ND                  | 17.4 ± 1.1           | 1.9 ± 0.8            |
| trf$hpx$                   | 6                   | -0.05†               | ND                  | 5.8 ± 0.9$^*$        | 27.4 ± 3.1†          |
| Daily mTf injections       | 5                   | 0.88 ± 0.25$^*$       | ND                  | 15.4 ± 3.1$^*$       | 6.5 ± 2.4$^*$        |
| Daily mTf-left for 1 wk    | 6                   | 0.13 ± 0.09$^*$       | ND                  | 14.6 ± 2.4           | 4.9 ± 3.0            |
| Daily hTf injections       | 5                   | <0.05$^*$             | 1.04 ± 0.6          | 14.9 ± 1.7$^*$       | 9.1 ± 4.5$^*$        |
| Daily hTf-left for 1 wk    | 5                   | <0.05$^*$             | <0.05               | 13.2 ± 1.1           | 11.2 ± 3.4           |
| Daily hAlb injections      | 3                   | <0.05                 | ND                  | 4.9 ± 1.4            | 22.9 ± 7.3           |

Data: mean ± SD for (n) determinations except for $^{**}$ where n = 6. Mouse (mTf) or human (hTf) transferrin, as appropriate, was assayed in the serum of noninjected or injected hypotransferrinemic mice.
Abbreviations: ND, not determined; hAlb, human albumin.
$^*P < .05$.
$^†P < .001$ as compared with the control group.
$^‡P < .03$.
$^¥P < .002$ as compared with untreated trf$hpx$ mice.
$^¥¥P < .002$ as compared with values in the appropriate 3-week-treated group.
both anemia and transferrin in the malregulation of intestinal iron absorption in homozygous hypotransferrinemic mice. The role of anemia/oxygen delivery in the regulation of iron absorption in trf<sup>hpx</sup> mice. Transfusion of erythrocytes from trf<sup>hpx</sup> animals resulted in a small, statistically insignificant increase in hemoglobin levels in recipient mice. Intestinal iron absorption values were unaffected. The lack of a significant effect on the hemoglobin level is surprising and may be attributable to the fact that erythrocytes from trf<sup>hpx</sup> animals are low in hemoglobin content. 5 Transfusion of washed erythrocytes from littermate controls (wild-type/heterozygotes), however, caused even after a single dose, a significant increase in the hemoglobin level and a decrease in the reticulocyte count. Multiple transfusion of erythrocytes led to further increases in hemoglobin, with the values being comparable with those seen in the control group. Even though mucosal transfer (as percentage of total uptake) decreased appreciably after the double and triple transfusions, total mucosal uptake failed to exhibit a significant decrease as compared with the untreated group. The decrease in mucosal transfer could not be attributable to either radioiron dilution within the duodenal mucosa (because non-heme iron levels were unaltered even after the triple transfusion) or to alterations in <sup>59</sup>Fe clearance by the liver. The decrease in mucosal transfer is in support of the findings of Buys et al. 10 However, our data do not reach the same conclusion (ie, normalized absorption values posttransfusions). This discrepancy may be attributable to differences in the absorption method used (ie, tied-off intestinal segment for 10 minutes v whole-body retention 4 to 24 hours after gavage) or the iron complex used (FeNTA v iron-ascorbate). Moreover, our absorption studies were confined to the duodenum, the primary region where iron absorption occurs, and were unaffected by effects on intestinal transit.

Erythrocyte transfusions had 3 effects, namely increased hemoglobin, decreased erythropoiesis, and increased liver iron (presumably because of exogenous/endogenous red cell breakdown). Any of these, independently, could perhaps have resulted in a decrease in mucosal transfer and overall iron absorption. The importance of the change in liver iron was tested by injecting iron dextran into trf<sup>hpx</sup> mice. No significant effect on iron absorption was seen even though liver iron levels were comparable with those in erythrocyte-infused trf<sup>hpx</sup> mice. Increased hemoglobin is presumed to affect intestinal iron absorption via oxygen delivery to the mucosa, as oxygen level in the inspired air is known to influence iron absorption. 26,27 However, exposure of homozygous mice to 40% oxygen for up to 3 days failed to alter iron absorption. Furthermore, intestinal mucosal <sup>31</sup>O<sub>2</sub> levels in trf<sup>hpx</sup> mice did not differ appreciably from those in the control group. Studies in pigs have shown that intestinal mucosal <sup>31</sup>O<sub>2</sub> levels remain fairly constant unless the hematocrit falls below 10%. 23 In previous studies, we found that acute alterations (≤1 week) in reticulocyte levels affected mucosal transfer more than mucosal uptake, 25 whereas chronic alterations (>1 week) in erythropoiesis affected mucosal uptake more than transfer. 11,12 Mice with chronic anemia and reticulocytosis comparable with trf<sup>hpx</sup> animals, namely β-thalassaemic mice, did not show the massively enhanced absorption of iron seen in trf<sup>hpx</sup> mice. 11 In an earlier study (unpublished observations, Simpson and Raja, January 1991) we found that exchange transfusion of blood (from the control group) into trf<sup>hpx</sup> mice modestly raised the hemoglobin levels and completely suppressed the reticulocyte response, without affecting iron absorption in these recipient mice. Based on these observations, it is unlikely that reticulocytosis is solely responsible for enhanced mucosal transfer and uptake in the trf<sup>hpx</sup> mice.

The tendency for the erythropoietic rate and iron absorption in genetically normal rodents to be decreased by hypertransfusion or hypoxia 26,27 and increased by bleeding 28 or hypoxia 26 is consistent with a regulatory mechanism, which is related to mucosal oxygen supply. In mice with lifelong anemia, as in genetic hypotransferrinemia, adaptive changes to the cardiovascular system and erythrocYTE oxygen affinity will have occurred to produce an animal able to maintain mucosal oxygen levels, as shown above, despite the anemia. We have previously shown that duodenal blood flux in homozygous animals is similar to that in the control group. 29 It is unsurprising that transfusion with erythrocytes from normal mice may decrease iron absorption, because it will increase oxygen delivery to the tissues. The finding that hypoxia does not depress iron absorption in these homozygous mice suggests that their adapted mucosal oxygen delivery system is, however, not responsive to increased inspired oxygen content. This highlights the fact that responses of trf<sup>hpx</sup> mice to experimental manipulation should be interpreted in light of their adaptation to chronic anemia.
Role of transferrin in the regulation of iron absorption in trf<sup>hpx</sup> mice. Transferrin, though clearly not obligatory for the absorptive process, may have a role in the regulatory process. Injection of purified mouse transferrin (1.0 to 4.4 mg) into trf<sup>hpx</sup> mice markedly increased delivery of <sup>59</sup>Fe to the bone marrow (and thence to red blood cells) and subsequently led to increases in the hemoglobin levels. The changes in <sup>59</sup>Fe incorporation by erythrocytes, which were apparent within 4 to 5 hours posttransferrin injection, were bigger with the higher transferrin doses; the radioiron clearance values peaked at 24 hours postinfusion and thereafter decreased with time and by 7 days had reverted to basal levels. Liver <sup>59</sup>Fe clearance values, in all cases but one, showed no significant reduction after transferrin injection(s). These findings show that the liver in trf<sup>hpx</sup> mice has a large capacity for clearance of nontransferrin bound iron, in support of previous findings. Erythrocytes, however, depend on transferrin as the main physiological route of iron delivery.

Although small decreases were apparent in the mucosal transfer of <sup>59</sup>Fe posttransferrin injection, the total mucosal uptake values did not change significantly in any of the treated groups except for the group given a double dose of 4.4 mg transferrin. The interpretation of the role of transferrin in the regulation of iron absorption is, however, hampered by the fact that hemoglobin values have increased concurrently. We conclude that iron absorption was reduced, but not normalized by acute correction of anemia, or by short-term injections of transferrin. We therefore investigated the involvement of transferrin in the control of iron absorption in trf<sup>hpx</sup> mice with long-term correction of anemia.

Transferrin (mouse/human) was injected daily for 3 weeks to normalize hemoglobin levels. This regime was calculated to produce transferrin levels which oscillated around a mean value equivalent to that seen in heterozygote mice (which show normal iron absorption rates). These transferrin-injected mice exhibited markedly reduced liver iron stores and had transferrin levels comparable with values seen in heterozygous mice. Iron absorption values (especially in the human transferrin-injected group) were considerably lower than those in the normally maintained, anemic trf<sup>hpx</sup> mice. Mice left un.injected thereafter for a week, thus allowing adequate time for the clearance of transferrin, but not for the development of anemia, showed markedly increased mucosal transfer and iron absorption values in spite of no changes being evident in the hemoglobin levels and liver iron stores; the total uptake values in both the mouse and human transferrin-treated groups were similar to those seen in normally maintained trf<sup>hpx</sup> mice. It is feasible that, in spite of normalized hemoglobin levels, low transferrin levels may lead to ineffective (iron-deficient) erythropoiesis, which may in turn contribute to the enhancement of iron absorption. The failure of daily mouse transferrin injections to completely normalize iron absorption is probably attributable to insufficient transferrin being present at all times throughout the experimental period. The finding that daily injections of human transferrin were more effective at normalizing absorption was apparently not attributable to the protein half-life, nor to a nonspecific effect of a foreign protein. It is possible that human transferrin interacts with transferrin-receptor in a way that more effectively regulates iron absorption.

These data show that the greatly enhanced intestinal iron absorption in trf<sup>hpx</sup> mice, leading to the highest degree of iron overload reported so far in mice fed a standard rodent diet, is not solely explained by anemia or increased production of erythrocytes. Transferrin levels do, however, influence iron absorption (especially mucosal transfer) independently of effects on hemoglobin levels.

Recently, a transgenic mouse colony with the homologous HFE gene disrupted has been established. These mutant mice were hematologically normal, had almost fully saturated transferrin, and showed hepatic iron-loading even though their diet was not supplemented with iron. It has previously been suggested that the amount of iron absorbed via the intestine is a function of the programming of crypt cells depending on body iron status. As HFe is reported to be present in the crypts and to interact with transferrin receptors, it is reasonable that HFe is involved in the sensing mechanism: Disruption of the gene would result in the crypts not being appropriately programmed, thus resulting in the malabsorption of iron through over expression of genes for iron absorption such as the recently described DCT1/Nramp2 transporter protein. Our data support the involvement of transferrin in the control of iron absorption possibly through direct effects on the intestinal mucosa.

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