The Kinetics of Iron Uptake by Isolated Intestinal Cells From Normal Mice and Mice With Sex-Linked Anemia

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No significant differences of $K_m$ or $V_{max}$ could be demonstrated for the initial rate (0-3 min) of iron uptake by preparations of isolated duodenal mucosal epithelial cells from normal or sex-linked anemic (s/a) mice. Isolated cells from s/a mice do, however, take up significantly more iron than normal cells after 20-min incubation. Upon reincubation with various types of mobilizing agents, the amount of iron released from, and the amount of iron remaining in, cells from s/a mice was significantly elevated compared to control values. The percentage of iron released was the same for both types of cells, for all mobilization media employed. The current data, in conjunction with previous studies that showed deficient mucosal to serosal transport of iron in everted duodenal sacs from s/a mice, suggest that the lesion in these mice may be located between the mucosal cell and the vascular compartment rather than in the mucosal cell.

A MAJOR SITE of regulation of iron metabolism is at the level of absorption in the duodenal mucosa. Iron absorbed from the lumen passes through the mucosal epithelial cells probably in the form of low molecular weight chelates and into the plasma. This process may be assisted by transferrin, the iron-transporting protein of the plasma, which could possibly serve, in its iron-depleted form (apotransferrin), as a "sink" for iron. However, this cannot be the sole regulating factor, since the absorption of iron in patients with sideroblastic or hemolytic anemia may be increased, despite high plasma iron concentrations and elevated iron stores. Also, patients with transferrinemia can absorb iron.

A paradoxical situation exists in mice with sex-linked anemia (gene symbol: s/a). These mice have elevated plasma transferrin concentrations, low iron stores, and low serum iron, but their intestinal absorption of ionic iron is impaired. In experiments with everted duodenal sacs from these mice, the transfer of iron from mucosal to serosal side is drastically decreased, although the amount taken up by the mucosa is approximately the same as normal. Excess iron, deposited probably as ferritin, can be seen in the luminal portion of duodenal mucosal epithelial cells in s/a mice. These cells have been considered to carry out the initial "entry" step of iron absorption appropriately, but the transfer of iron to the blood (i.e., serosal transfer or "exit" step) was thought to be defective. It has been suggested that this could be due to the lack of a "carrier" protein in the epithelial cells or to a deficient interaction of transferrin with the serosal membrane of these cells.

The "block" can be circumvented, however. Mice with sex-linked anemia have been shown to clear intravenously administered elemental iron from the plasma twice as fast as normal animals. Utilization of that iron for hemoglobin synthesis in s/a mice was found to be 2.5 times that of controls. This suggested that the lesion in s/a mice is distal to the vascular compartment.

If the lesion in s/a mice is a mucosal cell dysfunction, leading to enhanced deposition of iron in those cells, this may express itself as an elevated cellular rate of uptake for iron, a decreased mobilization of cellular iron by transferrin and/or apotransferrin, or both.

In the present study, a preparation that yields a high percentage of viable and predominantly individual duodenal mucosal cells from normal and s/a mice was used to examine the rates of uptake of iron. The release of iron from preloaded cells, with and without transferrin and apotransferrin in the medium, was also investigated.

MATERIALS AND METHODS

Animals

A colony of mice carrying the s/a gene (now in their 20th generation), repeatedly backcrossed to the C57BL/6J strain, is housed in plastic cages and maintained on "Rockland" complete mouse diet. The principles of laboratory animal care, as promulgated by the National Society of Medical Research, are observed in this laboratory.

Preparation of Isolated Intestinal Epithelial Cells

Isolated cells were prepared by a modification of the procedure reported previously for rat duodenum. Mice were killed by cervical dislocation and the small intestine quickly excised. The first 5 cm of the duodenum was everted with a glass capillary pipette, rinsed in ice-cold phosphate-buffered...
saline (PBS), then threaded over a zig-zag shaped Pasteur pipette, and tied in place with surgical thread. The entire assembly was shaken vigorously for a few seconds in cold PBS to ensure that no foreign material was retained on the everted gut.

The gut-pipette assembly was immersed in citrate solution at 37°C for 20 min. Four gut-pipette assembly were attached to a lucite block mounted on a vibromixer (Chemapac, Inc., Hoboken, N.J.). Cells were shed from the tissue by vibration in 35 ml of PBS/10 mM glucose (hereafter referred to as buffer) for 10 min at 37°C. The cells were then allowed to cool to 0–4°C, centrifuged at 750 g/5 min, and washed twice with cold buffer. Cells were resuspended in cold buffer, usually to the extent of 7.5 ml/duodenum. At this time, viability was estimated by trypan blue exclusion (0.1% in buffer); duration of viability was examined by oxygen consumption for periods up to 2 hr.

Iron Solutions

Iron solutions were prepared by dilution of a freshly made stock solution containing 10 mM ferrous ammonium sulfate and 20 mM sodium citrate in buffer. Radioactive iron, as 59Fe citrate (Mallincrodt), was added to the working solutions so that each sample tube would receive 75–100 nCi. All reagents were subsequently titrated to a pH of 7.00 with NaOH or HCl.

Iron Uptake Studies

Resuspended cells (0.5 ml) were aliquoted into polypropylene tubes and kept on ice until the start of the assay. The tubes were preincubated at assay temperature for at least 3 min. To start the reaction, an equal volume of iron solution (at the same temperature) was added, and the sample tubes were vortexed and returned to the shaking water bath. At termination, tubes were removed from the water bath, 3.0 ml ice-cold buffer was added, and the tubes were placed on ice. A duplicate pair of tubes for each ion concentration was kept at 0°C. Ice-cold iron solution was added to these tubes, and they were immediately diluted with 3.0 ml ice-cold buffer. After termination of the experiment, the cells were centrifuged at 750 g, resuspended, and washed twice with 3.0 ml ice-cold buffer, then resuspended with 1.0 ml buffer and counted in a Packard auto-gamma scintillation counter. For calculations, the amount of iron retained by the cells kept at 0°C was subtracted from that absorbed at 37°C.

This same protocol was used for experiments with varying pH (6.7, 7.0, 7.4, and 7.7), different buffers (TRIS, Imidazole, Pyrophosphate), and in the presence of various inhibitors (phloridzin, phloretin, rotenone, and dinitrophenol).

The possibility that apparent iron absorption observed in this system is artificial, and the result of the uptake of precipitated iron has been excluded by several experiments. First, the uptake of a nonabsorbable marker (57Co-cyanocobalamin) was less than 5% of the uptake of 59Fe under similar conditions. Second, upon ultrafiltration of the iron reagent through a diaflo membrane (Amicon Corp., Lexington, Mass.) with a mol wt cutoff of 10,000 daltons, no radioactivity was retained by the filter. Finally, no residual 59Fe could be detected in incubation tubes to which no cells had been added.

Kinetics of Iron Uptake

The kinetic parameters Kₚ and Vₘₐₓ were estimated using Lineweaver-Burk double reciprocal plots, as outlined for membrane transport.

Mobilization of Iron

The final cell pellets from the preparation of the cells from 4 normal and 4 sl/a mice were individually resuspended to 7.5 ml with buffer. The same volume of a radioiron solution was added (40 μM Fe). After 20 min of incubation at 37°C, the cells were harvested (vide supra). Duplicate aliquots of 0.5 ml were made from the resuspension and kept on ice until reincubation. Reincubation media were: albumin (0.5 mg/ml), transferrin (0.1 mg/ml), apotransferrin (0.1 mg/ml), and mouse serum (10%). Constituents were in buffer, which also served as a control medium. Cells were reincubated, then 0.5 ml of the appropriate reincubation medium was added, and the samples were returned to the shaking water bath. After 20 min, the reaction was stopped, cells were centrifuged, washed, and counted (vide supra), and compared to samples not reincubated, but processed equivalently.

Transferrin Preparation

Transferrin was prepared from serum of 50 normal C57BL/6J mice by the procedure of Baker et al. Transferrin was saturated with iron by overnight dialysis against 1.0 mM ferrous ammonium sulfate/2.0 mM sodium citrate/2.0 mM Na₃CO₃ in 25 mM PBS, pH 7.4. Subsequent dialysis against PBS (2 × 1000 ml) was done to remove any excess and nonspecifically bound iron. Apotransferrin was prepared by overnight dialysis against 0.5 M citrate, pH 5.8. Subsequent dialysis against PBS (2 × 1000 ml), pH 7.0, brought the protein back to assay conditions. Both preparations were checked for iron-binding capacity by measuring the difference in absorbance at 460 nm after addition of Fe₃-nitrilotriacetate (1:2, pH 7.0) using a Cary 118 recording spectrophotometer. No change was observed for the transferrin preparation; a large change (representing about 50% binding) was observed for the apotransferrin preparation.

All quantitative measurements were determined in reference to the amount of DNA in the sample, which was previously determined to be proportional to the number of cells.

Other

DNA was determined by a modification of the technique of Burton. Protein was assayed by the procedure of Lowry et al. Statistical analyses were done using Student's t test. Linear regression was analyzed using the method of least squares. Probability values for the computed regression coefficients and t statistics were taken from standard tables.
Fig. 2. Moles of iron absorbed in 3 min versus the amount of cells in medium (DNA content). Iron concentration was 2.5 μM. Cells were incubated at 0° and 37°C. The difference is plotted as the abscissa. Both sla (○) and normal (●) cells are plotted (r = 0.967; p < 0.001).

RESULTS

Viability

The appearance of the isolated cells is shown in Fig. 1. Some remained columnar, but most appeared rounded. About 90% retained their brush border. The preparation consumed oxygen linearly for at least 2 hr; trypan blue exclusion was in the range of 80%-90%.

Effects of Buffers, pH, and Inhibitors

TRIS, imidazole, and pyrophosphate buffer (25 mM, pH 7.0, in physiologic saline) inhibited iron uptake by the isolated cells, yielding 20.5%, 7.2%, and 1.0% of the amount of iron taken up after 10 min in the presence of phosphate buffer, respectively.

The initial rate of iron uptake was not influenced by changes of pH over a range of 6.7–7.7. There was no effect by the metabolic inhibitors rotenone and 2,6-dinitrophenol (25 μM), nor by the glucose transport inhibitors phloridzin or phloretin (25 μM).

Iron Uptake and Release

Iron uptake was directly proportional to the number of cells (Fig. 2) and was linear for both normal and sla cells for at least 3 min (Fig. 3). After 3 min, iron uptake slowed.

The initial uptake process appeared to have the characteristics of an enzyme or carrier-mediated process (Fig. 4). Double reciprocal plots of the data from individual experiments in Fig. 4 showed no significant differences for $K_m$ and $V_{max}$ between the two types of cells (Table 1).

Initial experiments showed that the amount of iron taken up in 10 min was a curvilinear function of the iron concentration in the medium (0.5–25 μM). More iron was taken up by sla cells than normal cells at all concentrations at this time point. The shape of the curves suggested an enzymatic or carrier-mediated process for both (Fig. 5).

After 20-min incubation, sla cells had taken up significantly more iron than normal cells (Table 2). After washing twice and reincubation of the two types of cells at 37°C, approximately 25%-30% of the iron originally taken up was released to media containing albumin, transferrin, or buffer alone. Apotransferrin and mouse serum effected the release of approximately 40% (Table 2). The absolute amount of iron released by normal cells reincubated with apotransferrin or serum was significantly greater than that released in buffer alone. Although a similar trend was observed for sla cells, statistical significance was not reached in similar comparisons due to the greater variability of
iron release by these cells. In normal cells, the percent of iron released in the presence of apotransferrin or serum was significantly elevated above that for cells reincubated in buffer. For sla cells, transferrin, apotransferrin, and serum effected a significantly higher percent of iron release than observed with buffer alone.

The percentage released was similar for both types of cells. However, sla cells retained and released significantly greater absolute amounts of iron than normal cells following reincubation, irrespective of the mobilizing agent employed (Table 2).

**DISCUSSION**

The initial uptake of iron by isolated mucosal cells does not appear to be an active transport process, since no inhibitory effect was produced by either rotenone or dinitrophenol. Furthermore, there does not appear to be any association between iron uptake and the well characterized sodium glucose transport system because no discernable effect was observed in the presence of phloretin or phloridzin.21-23

The present finding of a curvilinear relationship between the initial rate of iron uptake and the iron concentration of the medium suggests that the process may be one of facilitated diffusion.

Gitlin and Cruchaud26 showed that in mice two processes were involved in the absorption of iron from the gut in vivo. At higher dosages a first-order process was operable. At lower dosages a carrier-mediated process was involved.

Alternatively, Sheehan,24 using rat everted duodenal sacs, and Savin and Cook,13 using rat isolated duodenal epithelial cells, found a linear relationship between iron uptake (20 min) and iron concentration, suggesting a simple diffusion process. However, the experimental conditions and the animal species used by these workers differed from those used in the current investigation.

The initial rate of iron uptake, measured over the first 3 min of incubation, was not influenced by pH changes in the range of 6.7–7.7. Savin and Cook13 have observed a sharp increase in the amount of iron taken up in 20 min by both everted duodenal sacs and isolated cells from rats at pH 7.4. We did not investigate the possibility of pH effects at longer time periods.

There was no significant difference of $V_{max}$ or $K_m$ for
the initial rate of iron uptake between normal and sla cells. However, after 20-min incubation, significantly more iron was found in sla cells. The reason for this excess accumulation of iron by sla mucosal cells is uncertain. It might be due to the elevated levels of apoferritin and/or apoferritin synthesis in sla cells.25

Similar proportions of the radioiron originally taken up were released from both normal and sla mucosal cells. Although the rapidly and more slow turn over iron pools in sla cells appear to be increased in size, the ratio of these two pools seems to be about the same as in normal cells. The significantly higher net amount of iron released from sla cells does not suggest that the exit of iron from these cells is in any way impaired. Since incubation with apotransferrin was able to effect the release of equivalent percentages of iron from both normal and sla cells, and significantly more net iron from sla cells, the basic defect in sex-linked anemia does not appear to involve the interaction of circulating transferrin with mucosal cells.

The present studies fail to demonstrate any impairment in either the entrance into or exit of iron from the sla mucosal cell, in contrast to previous in vivo and in vitro studies showing a defect in the absorption of iron due to impaired mucosal to serosal transport of iron.5–12

There are two potential explanations for this apparent discrepancy. First, isolated mucosal cells in vitro, although viable, may not reflect the in vivo physiologic situation because correct orientation and cell-to-cell contact may be critical to their normal functioning. Second, the defect in sex-linked anemia may not reside

![Graph](image)

**Table 1. Kinetic Constants for Iron Uptake by Isolated Mucosal Cells**

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th></th>
<th>sla</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( V_{\text{max}} \times 10^{-8} ) (moles/min/mg DNA)</td>
<td>( K_{\text{m}} \times 10^{-8} ) (moles/liter)</td>
<td>( V_{\text{max}} )</td>
</tr>
<tr>
<td>1</td>
<td>0.20</td>
<td>1.30</td>
<td>0.18</td>
</tr>
<tr>
<td>2</td>
<td>0.26</td>
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<td>0.29</td>
</tr>
<tr>
<td>3</td>
<td>0.29</td>
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<td>0.27</td>
</tr>
<tr>
<td>4</td>
<td>0.22</td>
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<td>0.28</td>
</tr>
<tr>
<td>5</td>
<td>0.15</td>
<td>1.12</td>
<td>0.24</td>
</tr>
<tr>
<td>6</td>
<td>0.30</td>
<td>1.98</td>
<td>0.24</td>
</tr>
<tr>
<td>7</td>
<td>0.29</td>
<td>2.53</td>
<td>0.24</td>
</tr>
<tr>
<td>Mean</td>
<td>0.24</td>
<td>1.98</td>
<td>0.25</td>
</tr>
<tr>
<td>SD</td>
<td>±0.06</td>
<td>±0.70</td>
<td>±0.04</td>
</tr>
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\( p \) NS NS

The kinetic constants \( K_{\text{m}} \) and \( V_{\text{max}} \) for mucosal cell iron uptake were estimated by double reciprocal plots using the difference of cell pellet iron content between 0 and 3 min at 37°C as an estimate of the initial rate of uptake. Isolated mucosal epithelial cells from the duodenum of sla and normal mice were incubated with iron concentrations in the range of 0.5–10 \( \mu \)M. Each sample tube contained 75–100 nCi of \(^{59}\)Fe-citrate.
Fig. 5. Uptake of iron versus iron concentration for normal (□) and sla (○) cells incubated for 10 min. For details see Fig 2 and Methods. Data from a typical experiment is shown.

Table 2. Release of Radio-iron From Preloaded Isolated Mucosal Cells

<table>
<thead>
<tr>
<th></th>
<th>Original (20-min Incubation)</th>
<th>PBSG</th>
<th>ALB</th>
<th>TRF</th>
<th>APOTRF</th>
<th>Serum</th>
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<tr>
<td>Iron left in cells</td>
<td>Normal</td>
<td>Mean</td>
<td>5.28</td>
<td>5.15</td>
<td>5.27</td>
<td>5.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SD</td>
<td>±5.28</td>
<td>±5.15</td>
<td>±5.27</td>
<td>±5.60</td>
</tr>
<tr>
<td></td>
<td>sla</td>
<td>Mean</td>
<td>98.50</td>
<td>71.52</td>
<td>70.59</td>
<td>66.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SD</td>
<td>±21.91</td>
<td>±14.01</td>
<td>±13.89</td>
<td>±13.94</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ρ</td>
<td>.001</td>
<td>.001</td>
<td>.001</td>
<td>.01</td>
</tr>
<tr>
<td>Iron released (from original)</td>
<td>Normal</td>
<td>Mean</td>
<td>16.86</td>
<td>16.14</td>
<td>32.63</td>
<td>38.83</td>
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<tr>
<td></td>
<td></td>
<td>SD</td>
<td>±0.96</td>
<td>±1.72</td>
<td>±1.80</td>
<td>±1.83</td>
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<tr>
<td></td>
<td>sla</td>
<td>Mean</td>
<td>27.02</td>
<td>28.04</td>
<td>32.63</td>
<td>38.83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SD</td>
<td>±8.12</td>
<td>±8.52</td>
<td>±8.07</td>
<td>±12.38</td>
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<tr>
<td></td>
<td></td>
<td>ρ</td>
<td>.05</td>
<td>.025</td>
<td>.01</td>
<td>.05</td>
</tr>
<tr>
<td>Percent released</td>
<td>Normal</td>
<td>Mean</td>
<td>27.8</td>
<td>24.7</td>
<td>26.7</td>
<td>38.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SD</td>
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<tr>
<td></td>
<td>sla</td>
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<td>28.2</td>
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<td>38.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SD</td>
<td>±2.5</td>
<td>±2.5</td>
<td>±1.4</td>
<td>±4.2</td>
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</table>

Release of iron from preloaded cells. Isolated mucosal epithelial cells from the duodenum of sla and normal mice were loaded with 40 μM iron at 37°C for 20 min. The amount remaining after subsequent reincubation, in various media, was calculated. The amount released is the difference between that originally found in the pellet (postloading) and that found after reincubation (values are means ± SD, n = 4.)

*p < 0.01 (compared to PBSG).

*b*p < 0.001 (compared to PBSG).
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in the duodenal mucosal cell, but at some point by the recent observation of a yellow autofluorescent material in the duodenal lamina propria of mice with denudal vasculature. A defect at such a site could explain sex-linked anemia. However, it remains to be determined whether this material is the cause or the consequence of disordered intestinal iron absorption in sex-linked anemia.

REFERENCES

The kinetics of iron uptake by isolated intestinal cells from normal mice and mice with sex-linked anemia

JE Peppriell, JA Edwards and RM Bannerman