Effects of Iron-saturated Lactoferrin on Iron Absorption

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Iron absorption from iron-saturated lactoferrin was compared to that from ferrous sulfate in iron-deficient anemic rats. One group of rats was given 50 μg of iron orally once a day and changes in red blood cell density, hematocrit, and hemoglobin values were measured at 14-day intervals for 70 days. A statistically significant increase in these values was demonstrated for the rats fed iron-saturated lactoferrin (50 μg Fe/35 mg lactoferrin/day), while the ferrous sulfate group showed no improvement in these values. The results suggest that iron from iron-saturated lactoferrin is absorbed across the intestinal mucosa by a mechanism other than the one by which soluble iron salts are absorbed.

Lactoferrin (LF) is an iron-binding glycoprotein found in externally secreted fluids such as milk.1,2) Although many biological functions of LF have been clarified, its role in iron absorption is still under debate, as reviewed by Lönnerdal.3,4) The two side of the debate are as follows. (1) Iron from iron-saturated LF (Fe-LF) is not easily absorbed due to its low dissociation constant. (2) Iron from Fe-LF is easily absorbed because there are LF receptor sites on the intestines. The discrepancy is probably attributable to variations in experimental conditions such as the dose of iron, degree of iron saturation, source of LF, species of the experimental animal, and other dietary components. Fransson et al.5) have shown in an in vivo study using weaning mice that iron bound to LF is absorbed by the mice, when given to them at a level presently used in several infant formulas. Suckling pigs were also found to absorb iron from LF.6) Fransson’s results indicated that iron as Fe-LF was as easily absorbed as ferrous iron.5–7)

It is widely accepted that ferrous ions such as ferrous sulfate are more easily absorbed than other iron compounds,8) but since specific LF receptors were found on human9) and monkey10,11) brush borders there has been speculation that Fe-LF is more easily absorbed than ferrous irons. If a marginal dose level of iron could be found at which iron-deficient rats recover from anemia with Fe-LF but not with ferrous iron, this speculation would be confirmed.

In a preliminary experiment, iron-deficient rats were fed each day with zero to 200 μg of iron as ferrous sulfate or Fe-LF. The changes between the hemoglobin values at day zero and day 28 were examined to find the marginal dose level. Subsequently, anemic rats were orally administered the marginal dose of iron to see whether they could absorb iron more easily from Fe-LF.

MATERIALS AND METHODS

Lactoferrin. Bovine lactoferrin (LF) was prepared from skim milk by a one-step procedure through affinity chromatography using immobilized monoclonal antibodies against bovine LF.12) Briefly, 300 ml of the affinity gel (Affigel-10; Bio Rad Laboratories, CA, U.S.A.) immobilized with 3.0 g of antibodies was packed into a column (60 mm × 150 mm). After passing 10 l of raw skim milk through the column, components that did not interact with the antibodies were washed out with 0.5 M sodium chloride. Then LF was eluted with 0.2 M acetate buffer containing 0.15 M sodium chloride at pH 3.7. The eluate was immediately adjusted to pH 7.0 with 1 M sodium hydroxide, dialyzed against deionized water, and then lyophilized. Sodium dodecyl sulfate polyacrylamide gel
electrophoresis revealed that the LF preparation was 98% pure densitometrically.

Fe-LF was prepared by adding 20 ml of ferric chloride (FeCl₃·6H₂O) solution containing 10 mg Fe³⁺/ml to 21 of 1% LF solution. After stirring for one hour, the LF solution was dialyzed against an excess of deionized water for 48 hr. Apo-LF was prepared by subsequent dialysis against 0.1 M citrate (pH 2.2) containing 0.1% EDTA for 24 hr and then against an excess of deionized water for 72 hr. Both Fe-LF and apo-LF were lyophilized for storage. The iron content of LF was measured with a serum iron analysis kit (Wako Pure Chemical Industries, Ltd., Osaka). The degree of iron saturation was estimated from the iron content, assuming 100% saturation to be 1.43 μg Fe/ml LF (55.85 × 2/78,000). The iron content and saturation of apo- and Fe-LF are shown in Table I.

Diets. Iron-deficient and iron-supplemented (200 ppm Fe, ferric citrate) diets were purchased from Oriental Yeast Co., Ltd. (Tokyo). The formula of the iron-deficient diet is summarized in Table II. Minerals were measured on an atomic absorption spectrophotometer (Hitachi model 180-80, Tokyo). The iron-deficient diet contained 2.5 ppm of iron, less than 0.7 ppm of copper, and 6.7 ppm of zinc.

Animals and experimental design. Wistar female weanling rats (three weeks old) weighing 40 ~ 50 g were obtained from Charles River Japan Inc. (Atsugi, Kanagawa) and housed individually in plastic cages containing bedding in a temperature- and humidity-controlled room (22°C, 50%). Deionized water and diets were fed daily ad libitum. Anemia was induced in the rats by feeding them the iron-deficient diet containing 2.5 ppm iron for three weeks.

Measurement of RBC, Ht, and Hb. Samples of blood were collected into heparinized vessels from the caudal vein at 14-day intervals for 70 days. RBC, Ht, and Hb were measured on a Microcell counter CC-150A (Tao Iyou Denshi Co., Ltd., Kobe, Hyogo). Data were analyzed for statistical significance by Student's t-test.

Preliminary experiment. Thirty-two rats with Hb values below 7 g/100 ml were randomly divided into eight groups, and continued on the iron-deficient diet. Ferrous sulfate or Fe-LF were orally administered daily in the morning at levels of 10, 20, 50, and 200 μg of iron dissolved in 1 ml of deionized water. Changes in Hb values were examined at day zero and day 28 to find the marginal dose level.

Experiment at marginal dose level. Anemic rats (Hb value 8 g/100 ml) were randomly divided into four groups of eight rats, and continued on the iron-deficient diet. The experiment is shown in Table III. Each dose of iron was dissolved in 1 ml of deionized water and orally administered once a day for 70 days. Weight gains were recorded weekly. RBC, Ht, and Hb were measured at 14-day intervals.

Table I. Iron Content and Iron Saturation of Lactoferrins

<table>
<thead>
<tr>
<th></th>
<th>Fe-LF</th>
<th>apo-LF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron content (μg/mg LF)</td>
<td>1.47</td>
<td>0.02</td>
</tr>
<tr>
<td>Iron saturation (%)</td>
<td>103</td>
<td>1.4</td>
</tr>
</tbody>
</table>

* Iron saturation 100% = 1.43 μg Fe/mg LF.

Table II. Formula of the Iron-deficient Diet

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Contents (g/kg diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn starch</td>
<td>320</td>
</tr>
<tr>
<td>Sucrose</td>
<td>300</td>
</tr>
<tr>
<td>Casein</td>
<td>220</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>50</td>
</tr>
<tr>
<td>Cellulose powder</td>
<td>50</td>
</tr>
<tr>
<td>Mineral mixture*</td>
<td>40</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>20</td>
</tr>
</tbody>
</table>

* Composition of mineral mixture (g/kg diet): Ca lactate, 14.5; KH₂PO₄, 11.4; Na₂HPO₄, 1.8; CaHPO₄·2KH₂PO₄, 4.1; MgSO₄·7H₂O, 3.0; NaCl, 2.0; KCl, 3.2.

b Composition of vitamin mixture (mg/kg diet): retinyl palmitate (500,000 IU), 20; thiamin HCl, 80; riboflavin, 400; pyridoxine HCl, 80; ascorbic acid, 3,000; cholecalciferol (400,000 IU), 5; α-tocopherol (50%), 600; menadione, 20; niacin, 600; Ca pantothenate, 500; inositol, 600; folic acid, 20; choline chloride, 1,000; cellulose powder, 13,035.

Table III. Experimental Design

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample</th>
<th>n</th>
<th>Administration level</th>
<th>Administered iron (μg/day)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>apo-LF</td>
<td>8</td>
<td>35 mg/ml/day</td>
<td>0.7</td>
</tr>
<tr>
<td>2</td>
<td>Fe-LF</td>
<td>8</td>
<td>35 mg/ml/day</td>
<td>51.5</td>
</tr>
<tr>
<td>3</td>
<td>FeSO₄·7H₂O</td>
<td>8</td>
<td>250 μg/ml/day</td>
<td>50.0</td>
</tr>
<tr>
<td>4</td>
<td>H₂O</td>
<td>8</td>
<td>1 ml/day</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>H₂O</td>
<td>8</td>
<td>1 ml/day</td>
<td>0</td>
</tr>
</tbody>
</table>

* Rats were fed ad libitum with iron-deficient diet containing 2.5 ppm of iron. One ml of sample with the given amount of iron was orally administered once a day.

b Control group was fed ad libitum with iron-supplemented diet containing 200 ppm of iron, and orally administered one ml of deionized water once a day.
RESULTS

1. Preliminary experiment

Table IV shows the results of the preliminary experiment on Hb values on day zero and day 28 when anemic rats were administered ferrous sulfate or Fe-LF at iron levels of zero, 10, 20, 50, and 200 µg/day. Rats administered ferrous sulfate or Fe-LF at iron levels of 10 and 20 µg/day showed no increase in Hb values. At an iron level of 50 µg/day, ferrous sulfate did not increase Hb values, but a significant increase was demonstrated with Fe-LF. Ferrous sulfate increased Hb values at an iron level of 200 µg/day. The marginal level of orally administered iron was found to be 50 µg/day, although the statistical significance was uncertain because the population was too small.

2. Experiment at marginal dose level

As shown in Fig. 1, weight gains of rats were not significantly influenced by the diets and administered samples. The mean weight gain during the experimental term was 126 g.

Changes in RBC are shown in Fig. 2. The RBC values of the control group fluctuated between 8 and 9 × 10^6 cells/µl during the experimental term, but the apo-LF and the deionized water groups had about 3 × 10^6 cells/µl. The RBC level of the Fe-LF group (7.1 ± 0.5 × 10^6 cells/µl) reached the same level as the control group (7.9 ± 0.4 × 10^6 cells/µl) on day 14, and exceeded the level of the control group on day 56. In contrast, little increase

<table>
<thead>
<tr>
<th>Administered samples (µg Fe/day)</th>
<th>Day 0</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized water (0)</td>
<td>6.3 ± 0.4</td>
<td>3.5 ± 0.6</td>
</tr>
<tr>
<td>FeSO₄·7H₂O (10)</td>
<td>5.9 ± 0.5</td>
<td>3.9 ± 0.3</td>
</tr>
<tr>
<td>FeSO₄·7H₂O (20)</td>
<td>5.9 ± 0.6</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>FeSO₄·7H₂O (50)</td>
<td>5.9 ± 0.5</td>
<td>5.2 ± 0.6</td>
</tr>
<tr>
<td>FeSO₄·7H₂O (200)</td>
<td>6.2 ± 0.8</td>
<td>10.8 ± 1.1</td>
</tr>
<tr>
<td>Fe-LF (10)</td>
<td>5.9 ± 0.7</td>
<td>4.6 ± 0.7</td>
</tr>
<tr>
<td>Fe-LF (20)</td>
<td>5.8 ± 0.4</td>
<td>5.9 ± 0.5</td>
</tr>
<tr>
<td>Fe-LF (50)</td>
<td>5.8 ± 0.6</td>
<td>8.5 ± 0.9</td>
</tr>
</tbody>
</table>

Values shown represent means ± SD for four rats in each group.

Fig. 1. Growth Curves of Rats.
Points represent the mean body weight of eight rats orally administered apo-LF 35 mg (○), Fe-LF 35 mg (●), FeSO₄·7H₂O 250 µg (△), or one ml of deionized water (▲) in iron-deficient diet groups, and one ml of deionized water (□) in iron-supplemented diet group. Vertical lines indicate standard deviation.

Fig. 2. Changes in Red Blood Cell Density.
Points represent the mean red blood cell density of eight rats orally administered apo-LF 35 mg (○), Fe-LF 35 mg (●), FeSO₄·7H₂O 250 µg (△), or one ml of deionized water (▲) in iron-deficient diet groups, and one ml of deionized water (□) in iron-supplemented diet group. Vertical lines indicate standard deviation.
was detected in the ferrous sulfate group and the value was $6.1 \pm 1.3 \times 10^6$ cells/$\mu l$ on day 70. Figure 3 shows changes in Ht percentages.

The Ht value of the Fe-LF group reached the same level as the control group on day 56, and was more than 60% on day 70. The Ht value of the ferrous sulfate group increased slightly, but not significantly, between day zero and day 70.

Similar changes in Hb were observed, as shown in Fig. 4. The Hb values of the control group fluctuated between 15 and 16 g/100 ml, while those of the apo-LF and deionized water groups gradually decreased to about 3 g/100 ml. Although only a slight increase in Hb value was observed in the ferrous sulfate group, the Hb value of the Fe-LF group increased greatly and reached the level of the control group.

**DISCUSSION**

Ferrous sulfate is the compound most commonly used in the oral treatment of iron deficiency because it is believed to be more readily absorbed than other iron compounds.\(^5,15\) The absorption of soluble iron salts is affected by other compounds in the diet.\(^6\) Our experiment showed a marked increase in Hb value with ferrous sulfate at an iron dose level of 200 $\mu g$/day, but not at 50 $\mu g$/day. To confirm whether Fe-LF is more easily absorbed than ferrous iron, the effectiveness of Fe-LF was compared with that of ferrous sulfate at the marginal dose level of iron (50 $\mu g$ Fe/day).

Significant increase in RBC, Ht, and Hb values of iron-deficient rats fed 35 mg of Fe-LF (51.5 $\mu g$ of iron) a day clearly demonstrated that iron was more easily absorbed from Fe-LF than from ferrous iron, as suggested by Fransson et al.\(^5,6\)

McMillan et al.\(^17\) showed that humanized infant formula fortified with Fe-LF administered to adult humans did not increase iron absorption. However, under their experimental conditions, it is possible that iron was present in an inorganic form and may have become complexed to other compounds in the formula. Fairweather-Tait et al.\(^18\) suggested that native human LF (11% iron-saturated) and bovine (14% iron-saturated) had no effect
on iron absorption in anemic rats. De Vet and Van Gool\textsuperscript{(9)} found a negative correlation between iron absorption and duodenal concentration of LF in adult humans. De Laey et al.\textsuperscript{(20)} studied the effects of human LF on iron absorption using everted duodenal sacs from rats and guinea pigs, and concluded that exogenous apo-LF significantly decreased the mucosal iron uptake, while no significant iron uptake in the mucosal cells was observed with Fe-LF. These groups suggested that LF may protect the intestinal mucosa from excess absorption of the iron by binding to iron in the intestinal tract and thus making it unavailable. However, these negative results may be attributable to experimental conditions such as the dose level of iron, degree of iron saturation, origin of LF, and species of the experimental animals. To evaluate the promoting effects of LF on iron absorption, it is conceivable that the administration level of iron and the degree of iron saturation with LF are of most importance. Also, it is indicated that there may be a difference between heterologous and homologous LF.\textsuperscript{(9)} This possible species effect should be investigated even though the chemical properties of LF from different species appear to be very similar.

Although the mechanism by which iron is absorbed from Fe-LF is still uncertain, the ease of its absorption might be due to the high solubility of iron bound to LF and/or to the specific absorption mechanism of Fe-LF. If the former is the case, Fe-LF should be enzymatically hydrolyzed to small peptides, still having iron-binding capability. However, it is considered that Fe-LF is not completely destroyed by digestive enzymes\textsuperscript{(21) - (23)} and retains its iron under acidic conditions.\textsuperscript{(24, 25)} Suzuki et al.\textsuperscript{(21)} suggested that LF was considerably resistant to pepsin in the stomach of neonates, because the pH value of the stomach contents rose above 4.5 when newborn infants drank milk. The finding of significant amounts of intact LF in the feces of breast-fed infants is consistent with the high resistance of LF to proteolytic hydrolysis in the small intestine.\textsuperscript{(26)} Similar results were obtained with rats, since intact LF was detected by immunodiffusion of fecal extracts from rats that had been given Fe-LF using antiserum against bovine LF (data not shown). The iron binding sites of LF are made three-dimensionally by two tyrosines, one histidine, and one aspartate,\textsuperscript{(27)} so it is not likely that conformationally altered LF retains iron after hydrolysis.

It is widely accepted that LF interacts with a variety of cells such as macrophages\textsuperscript{(28, 29)} and neutrophils.\textsuperscript{(30, 31)} The mechanism of the interaction is unclear so far, but iron from Fe-LF is thought to be transported across the cells through specific LF receptors. This study suggested that iron from Fe-LF was absorbed across the intestinal mucosa by an alternative mechanism from the transport for soluble iron salts. Intensive work is needed to explore the role of LF in the absorption of iron.

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\textbf{REFERENCES}