Iron Deficiency and Marginal Vitamin A Deficiency Affect Growth, Hematological Indices and the Regulation of Iron Metabolism Genes in Rats

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ABSTRACT Iron deficiency and marginal vitamin A (VA) deficiency frequently coexist and affect billions of people, mostly children and women, worldwide. The effects of these micronutrient deficiencies alone and in combination on hematologic, biochemical and molecular indices of iron and VA status were investigated in a 2 × 2 randomized blocked study conducted in growing male Sprague-Dawley rats. From 3–8 wk of age, rats were fed one of four purified diets that were either adequate or restricted in iron (Fe) and adequate or marginal in VA: "Fe"VA, 20.3 and 0.367 μg/g, respectively, denoted control diet; "Fe"VA, 3.34 and 0.405 μg/g; "FeVA", 22.2 and 0.051 μg/g; or "FeVA", 3.03 and 0.055 μg/g. Weight-matched rats fed adequate micronutrients were included to control for possible confounding effects of Fe deficiency on growth and feed efficiency. Iron restriction reduced (P < 0.05) weight gain, feed efficiency, blood hemoglobin and hematocrit. Plasma and liver iron and plasma transferrin saturation were reduced by −50%, whereas liver transferrin mRNA and protein and transferrin receptor mRNA were elevated, as was liver ferritin light-chain protein and light-chain mRNA. Liver heavy-chain ferritin mRNAs, hemopexin, ceruloplasmin and cellular retinol-binding protein mRNA were not affected by iron or VA restriction. Although marginal VA deficiency did not exacerbate indices of poor iron status during iron deficiency, iron deficiency was associated with lower plasma retinol and elevated liver VA concentrations. These results are consistent with an impaired mobilization of liver retinol during iron deficiency as well as multiple alterations in iron metabolism. J. Nutr. 132: 3607–3615, 2002.

KEY WORDS: • iron deficiency • marginal vitamin A deficiency • transferrin • ferritin • rats

Iron deficiency anemia is estimated to affect ~30% of the world’s population (1,2). In many parts of the world, iron deficiency coexists with deficiencies of other micronutrients, including vitamin A (VA)3. Although new cases of severe VA deficiency (xerophthalmia) still occur each year (3), it is estimated that mild, or marginal, VA deficiency is far more prevalent, especially among children, pregnant and lactating women in the developing world, and may affect as many as 250 million people worldwide (2).

As early as the 1920s, VA deficiency and anemia or other indices of low iron status were reported to be associated in humans and animals (4–6). In observational studies, Mejia et al. (7) reported an association of low hemoglobin (Hb) and low plasma iron with low plasma retinol in children. Transferrin saturation (%TS) was significantly correlated with retinol levels (7) but there was no association of plasma retinol and total iron-binding capacity (TIBC) (7). Overall, serum retinol has been shown to be positively associated with Hb (7–10), hematocrit (Ht) (8,9,11,12), and serum iron (7–12) and %TS (7–10,12,13). From these studies, VA has been proposed to play a role in regulating plasma iron levels, with VA deficiency decreasing plasma iron, resulting in decreased %TS. In experimental studies conducted in adult men with very low VA intakes for 359–771 d, Hodges et al. (14) observed the unexpected development of moderate anemia (low Hb, Ht and RBC counts), which did not improve with medicinal iron supplementation during VA deficiency, but was ameliorated when VA was given in addition to iron supplements. These results suggested that iron stores were unavailable for hematopoiesis during VA deficiency, implying that VA plays a role in the release of iron from the liver. In cross-sectional studies and VA supplementation trials conducted to determine the relationship between VA and iron in women and children in susceptible populations, VA significantly increased serum retinol (8), as well as Hb (8,9,15–18), Ht (18), erythrocyte counts (8), serum iron (8,19,20) and %TS (8,19), although the increase in Hb tended to be greatest after supplementation with iron and VA in combination (8). In general, there was no effect of VA supplementation on TIBC or serum ferritin, with the exception of a study in which serum ferritin was increased by VA supplementation (16). The rat has been used since the 1920s as a model in which...
to investigate the relationship between VA deficiency and iron deficiency (5). Hematopoiesis, measured as the incorporation of $^{59}$Fe into RBC, was reduced by 40–50% in VA-deficient rats (21), whereas liver and spleen iron increased (14,21–23). Both Hb and Ht were lower in rats with either marginal (22) or more severe VA deficiency (23). However, in other studies, hematologic signs of anemia during VA deficiency were not observed (14,24). Conversely, after VA supplementation of VA-deficient rats, plasma iron and TIBC increased, whereas iron concentrations in the liver, kidney, spleen and tibia decreased (25). From these data, it has been hypothesized that VA deficiency promotes the sequestration of iron in tissues, making it unavailable for erythropoiesis, resulting eventually in anemia and abnormal iron indices.

In the present study, we tested the independent and combined effects of iron deficiency and marginal VA deficiency on hematologic and molecular indices of iron and VA status. The design of our study differs in several ways from previous studies. First, we used a model of marginal VA deficiency, due to its prevalence in human populations, rather than severe VA deficiency. The diet contained marginal VA (VA$_{m}$) forming to contain enough VA to produce a moderately low plasma retinol concentration without accumulation of VA in liver (26). Second, weaning rats were used to model the period of rapid growth when effects of both micronutrient deficiencies may develop rapidly in children (2,27). Third, we designed our study to investigate the effects of an iron-adequate diet that would support normal growth but not result in high tissue stores, vs. an iron-restricted diet (3.0–3.3 μg/g diet) that was expected to induce anemia (28). The iron-restricted diet was also expected to reduce growth and the efficiency of feed utilization (29). In some previous studies using micronutrient-deficient diets, body weight gain has been used as a covariate to control statistically for the effects of micronutrient deficiency on growth (30). Although we took this approach in the present study, we also included two groups of individually weight-matched (WM) rats that were fed restricted amounts of diet containing adequate amounts of iron and VA. The comparison of these WM groups with their respective iron-restricted groups provided additional assurance that effects in the main study that we have attributed to iron deficiency were not the result of growth restriction per se. Finally, with respect to VA, we chose a marginal level to provide enough of this micronutrient for adequate growth, but not enough for liver VA storage. We anticipated that by incorporating these design features, our study would provide a stringent test of the independent effects of low dietary iron, marginal dietary VA and the interactions between them under nonexaggerated dietary conditions.

MATERIALS AND METHODS

Animals. Animal procedures were approved by The Pennsylvania State University’s Animal Care and Use Committee. Four lactating Sprague-Dawley rats with 10 male pups each were purchased from Charles River Breeding Laboratory (Wilmington, MA). The experiment was divided into three blocks (A, B and C) separated by 2 d each to allow sufficient time on the last day of the study for tissue collection. Environmental conditions were controlled (24-h light: dark cycle; 25°C) and distilled deionized water was provided continuously. Upon arrival, the dams were fed a VA-free diet (31); this strategy does not result in VA deficiency but does reduce the quantity of milk VA ingested by the pups, thereby facilitating the development of marginal VA deficiency after weaning (32,33). When the pups were 21 d old, they were randomly separated into individual stainless steel wire hanging cages, which were randomly arranged within the cage rack, and fed their respective diets. At the beginning of the experimental period, there were no differences in body weight among the four experimental groups in the 2 × 2 study or the two WM groups (data not shown).

Diets and feeding. The basic AIN93 diet (34) was modified as shown in Table 1 and tested for iron and VA (retinol; see legend to Table 1). Two additional groups of WM rats were fed a micronutrient adequate diet (see below) in amounts that kept the weight of the WM rats equal to the weight of the paired rats fed either the FeVAm$_1$ or FeVAm$_2$ diet. All rats in the 2 × 2 study consumed food ad libitum. By 2 wk after commencement of treatment, the rats fed either iron-deficient diet exhibited a reduction in food intake and feed efficiency, as expected (29), and the quantity of the control (FeVAm) diet fed to the WM rats was restricted accordingly. Because weight matching also resulted in an ~10% reduction in intake of micronutrients, we prepared a new diet for the WM groups which contained 29.6 μg/g of Fe and 1.29 μg/g of VA to compensate and to ensure adequate micronutrient intakes. This diet was fed to the WM groups for the last 2 wk of the 5-wk feeding period.

Tissue and blood collection. After overnight food deprivation, rats were killed by carbon dioxide asphyxiation and blood was collected from the vena cava into heparinized syringes or hematocrit tubes for immediate Hb and Ht analysis (28). The plasma was stored at −20°C for later iron and VA analyses. The wet weights of the liver and spleen were recorded and tissues were rapidly frozen in liquid nitrogen, then stored at −70°C for future analyses.

Iron indices. At the beginning of the feeding study, tail blood was collected from six randomly chosen rats at weaning to determine baseline Hb and Ht values. After 2 wk, tail bleeding was conducted weekly to monitor plasma Hb and Ht values, measured as above.

At the end of the 5-wk feeding period, plasma iron and TIBC were determined by a colorimetric method (35). Pooled plasma samples were used as internal standards for both plasma iron and TIBC analysis on each microtiter plate to control for interassay variability. Transferrin saturation was determined by calculating the ratio of plasma Fe divided by TIBC and expressed as a percentage (%TS). Liver nonheme iron for individual rats was measured after ~48 h acid hydrolysis using a previously described colorimetric method (35).

Vitamin A. Liver and plasma retinol concentrations, and the VA contents of the diets were measured, after saponification and extraction, by HPLC as previously described (36). Because small variances were expected for tissue VA concentrations, we reduced the number of samples for analysis by pooling equal amounts of the two samples from the same diet group in each block (A, B, C).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/kg diet</th>
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<tbody>
<tr>
<td>Cornstarch$^1$</td>
<td>629.5</td>
</tr>
<tr>
<td>Casein, low trace element</td>
<td>200.0</td>
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<tr>
<td>Fiber (Alphacel)</td>
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<tr>
<td>Soybean oil$^4$</td>
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</tr>
<tr>
<td>Mineral mix (AIN-93G-MX)$^3$,</td>
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</tr>
<tr>
<td>Vitamin mix (AIN-93-VX)$^5$</td>
<td>10.0</td>
</tr>
<tr>
<td>L-Cystine</td>
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</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.50</td>
</tr>
<tr>
<td>tert-Butylhydroquinone</td>
<td>0.014</td>
</tr>
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</table>

1 The original diet (34) called for cornstarch, dextrinized cornstarch and sucrose as the sources of carbohydrates.

2 Vitamin A (VA), when added, was added as retinol palmitate with the oil; see footnote 3.

3 Results of diet analysis for iron (Fe) and VA were (μg/g): FeVAm$_1$, 20.3 and 0.367; FeVAm$_2$, 3.34 and 0.405; FeVAm, 22.2 and 0.051; FeVAm, 3.03 and 0.055.

4 Mineral mix was adjusted for different levels of iron (2.77 mg ferric citrate for iron-adequate, no added ferric citrate for iron-deficient diet); see footnote 3.

5 Vitamin A was omitted.
RNA analysis. The relative mRNA expression was analyzed for transferrin, ferritin light chain, ferritin heavy chain, hemopexin, ceruloplasmin (Cp) and cellular retinol-binding protein (CRBP). RNA was extracted and Northern blots were prepared using 15 μg RNA/lane, on each of four replicate gels, by standard methods described in detail (37). The cDNA fragments used as probes were obtained for ferritin light chain (489 bp) from Dr. Richard Eisenstein (University of Wisconsin, Madison, WI), and for ferritin heavy chain (388 bp) from Dr. James Connor (Hershey Medical Center, Hershey, PA). The probe for CRBP was kindly donated by Dr. Reza Zolfaghari, the probe for Cp was made in our laboratory previously (38). The probe for hemopexin was prepared by Dr. Hongyu Zhang. Blots were exposed to storage phosphor screens on Quick Spin G-25 Sephadex columns (Boehringer Mannheim, Indianapolis, IN). Blots were exposed to storage phosphor screens by Dr. Reza Zolfaghari, the probe for Cp was kindly donated by Dr. Hongyu Zhang. After exposure, the blots were stripped of the DNA probe, exposed to film after stripping probe to detect any remaining radioactive probe, reproduced to detect 18S RNA expression and quantified again. Data for each experiment were adjusted for the 18S rRNA signal from the same lane. Afterwards, values were divided by the mean signal-to-18S ratio for the Fe VA group (normalized to 1.0) before statistical analysis.

Protein analysis. ELISA was utilized to determine relative protein levels of transferrin, transferrin receptor, and ferritin light chain, using methods described previously (39,40). Liver homogenates were pooled for the two rats in each block (3 pools/treatment) for analysis, using methods described previously (39,40). Liver homogenates and ferritin heavy chain (388 bp) from Dr. James Connor (Hershey Medical Center, Hershey, PA) and ferritin light chain (489 bp) from Dr. Richard Eisenstein (University of Wisconsin, Madison, WI) were used as probes. The cDNA fragments used as probes were obtained for transferrin, ferritin light chain, ferritin heavy chain, hemopexin, ceruloplasmin (Cp) and cellular retinol-binding protein (CRBP). RNA was extracted and Northern blots were prepared using 15 μg RNA/lane, on each of four replicate gels, by standard methods described in detail (37). The cDNA fragments used as probes were obtained for transferrin light chain (489 bp) from Dr. Richard Eisenstein (University of Wisconsin, Madison, WI), and for ferritin heavy chain (388 bp) from Dr. James Connor (Hershey Medical Center, Hershey, PA). The probe for CRBP was kindly donated by Dr. Reza Zolfaghari, the probe for Cp was kindly donated by Dr. Hongyu Zhang. After exposure, the blots were stripped of the DNA probe, exposed to film after stripping probe to detect any remaining radioactive probe, reproduced to detect 18S RNA expression and quantified again. Data for each experiment were adjusted for the 18S rRNA signal from the same lane. Afterwards, values were divided by the mean signal-to-18S ratio for the Fe VA group (normalized to 1.0) before statistical analysis.

Protein analysis. ELISA was utilized to determine relative protein levels of transferrin, transferrin receptor, and ferritin light chain, using methods described previously (39,40). Liver homogenates were pooled for the two rats in each block (3 pools/treatment) for analysis, conducted in triplicate. External standards of rate apo-transferrin and liver ferritin type IV were purchased (Sigma Chemical, St. Louis, MO) for the respective ELISA assays, and rat transferrin receptor protein levels were expressed relative to control levels.

RESULTS

Growth. Rats fed either of the iron-deficient diets (Fe VA and FeVAm) grew more slowly than rats fed the control (Fe VA) diet, as evidenced by lower absolute body weight at the end of the study (Table 2), and the lower percentage weight gain (not shown). Growth retardation was significant (P < 0.01) in both iron-deficient groups compared with the iron-adequate groups by wk 2 of dietary treatment (data not shown). The growth of rats in the two WM groups, which were not restricted in iron, did not differ from that of the iron-deficient rats to which they were paired (Table 2). As expected, marginal VA status did not reduce growth or feed efficiency. Food intake and feed efficiency were reduced in the iron-deficient groups, regardless of dietary VA (Table 2), as anticipated from previous studies (29).

At the end of the study, there was a significant reduction in absolute but not in relative liver weight in the iron-restricted rats (Table 3). The absolute spleen weight was 40–60% higher in the iron-deficient groups compared with the iron-adequate groups. This may be the result of scavenging by the splenic red pulp macrophages of RBC, which did not mature properly due to the lack of iron (25,30).

Progression of iron deficiency and indices of tissue iron status. The progression of iron deficiency was monitored throughout the 3-wk experimental period by measuring blood Hb concentration (Fig. 1A) and Ht (Fig. 1B). In the iron-restricted groups, both indices declined within 2 wk, and there was no additional effect of VA status.

Plasma iron concentrations (Fig. 2A) and %TS (Fig. 2B) showed a more pronounced pattern because values were reduced by iron restriction and marginal VA deficiency, but there was no interaction (P = 0.037–0.32). Overall, both values decreased progressively: control group > FeVAm > Fe VA > FeVAm (Fig. 2). The TIBC values (means ± SD) were 97 ± 3, 99 ± 3, 102 ± 3 and 103 ± 1 μmol/L in the Fe VA, FeVAm, Fe VA, and FeVAm groups, respectively, and were not different. Liver iron was also reduced in rats fed the iron-restricted diets, as expected, when expressed both as a concentration (Fig. 2C) and as total liver iron (data not shown). In neither case did VA affect liver iron, and there was no interaction.

Vitamin A status. Both dietary iron (P = 0.0001) and VA (P = 0.005) affected plasma retinol, but there was no interaction (P = 0.67). Overall, plasma retinol decreased progressively: control > FeVAm > Fe VA > FeVAm (Fig. 3A). Dietary iron and VA (both P = 0.0001), and their interaction

<table>
<thead>
<tr>
<th>Table 2</th>
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<tr>
<td><strong>Table 2</strong> Body weight, food intake and feed efficiency of rats fed iron-restricted (–Fe) and vitamin A-marginal (VA m) diets for 5 wk, and of iron-adequate rats weight-matched (WM) to the iron-restricted groups1</td>
</tr>
<tr>
<td>---------------------------------</td>
</tr>
<tr>
<td>Diet</td>
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<tr>
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<tr>
<td>g</td>
</tr>
<tr>
<td>g gain/g feed</td>
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<tr>
<td>WM iron-adequate</td>
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<tr>
<td>groups:</td>
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<tr>
<td>WM, Fe VA</td>
</tr>
<tr>
<td>WM, FeVAm</td>
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</tbody>
</table>

1 Values are means ± SEM, n = 6. Means in a column without a common letter differ, P < 0.05. * Different from rats fed the Fe-restricted diet indicated, P < 0.05.
Liver retinol was slightly higher ($P < 0.05$) in the FeVAm group than in the Fe VA group, and the Fe VA group also exceeded the Fe VA group. Similar results were obtained for liver VA contents (not shown).

### Gene expression of iron-transport and storage proteins.

A scanned image of a representative Northern blot for liver transferrin mRNA is shown in Figure 4A, with the 18S rRNA control. The relative expression of transferrin mRNA in liver was elevated in iron-restricted rats regardless of their VA status (Fig. 4B). Marginal VA deficiency did not affect transferrin mRNA expression. Transferrin protein levels in the liver were also elevated in iron-restricted rats regardless of VA

<table>
<thead>
<tr>
<th>Diet</th>
<th>Liver g</th>
<th>Liver g/100 g body</th>
<th>Spleen g</th>
<th>Spleen g/100 g body</th>
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</thead>
<tbody>
<tr>
<td>$^{+}$Fe $^{+}$VA</td>
<td>9.45 ± 0.40b</td>
<td>3.49 ± 0.08</td>
<td>0.79 ± 0.03b</td>
<td>0.29 ± 0.01b</td>
</tr>
<tr>
<td>$^{+}$FeVAm</td>
<td>10.61 ± 0.57c</td>
<td>3.50 ± 0.08</td>
<td>0.72 ± 0.05b</td>
<td>0.24 ± 0.01a</td>
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<tr>
<td>$^{-}$FeVA</td>
<td>5.13 ± 0.29a</td>
<td>3.21 ± 0.15</td>
<td>0.49 ± 0.01a</td>
<td>0.31 ± 0.01b</td>
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<tr>
<td>$^{-}$FeVAm</td>
<td>4.72 ± 0.19a</td>
<td>3.35 ± 0.14</td>
<td>0.47 ± 0.02a</td>
<td>0.33 ± 0.01b</td>
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<td>WM iron-adequate groups:</td>
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<tr>
<td>WM to $^{-}$Fe $^{+}$VA</td>
<td>5.92 ± 0.27</td>
<td>3.56 ± 0.12</td>
<td>0.40 ± 0.02*</td>
<td>0.24 ± 0.01*</td>
</tr>
<tr>
<td>WM to $^{-}$FeVAm</td>
<td>5.46 ± 0.18</td>
<td>3.62 ± 0.15</td>
<td>0.32 ± 0.01*</td>
<td>0.22 ± 0.01*</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, $n = 6$. Means in a column without a common letter differ, $P < 0.05$. * Different from rats fed the Fe-restricted diet indicated, $P < 0.05$.

**FIGURE 1**  Hemoglobin (A) and hematocrit (B) in postweaning rats during the development of iron deficiency, marginal vitamin A (VA) deficiency and their combination. Initial values were measured on d 0 in 6 randomly selected rats before assignment to experimental diets; $n = 6$ /group except for the $^{-}$Fe VA group on d 36, $n = 5$. Data are presented as the mean ± SEM. For both Hb and Ht, there was an effect of dietary iron ($P < 0.0001$, 2-way ANOVA) by d 14 and at each time thereafter. Asterisks indicate groups that differed significantly ($P < 0.05$) from the control group. Abbreviations: $^{+}$Fe, iron adequate; $^{-}$Fe, iron deficient; $^{+}$VA, vitamin A adequate; VAm, VA marginal. Values for weight-matched rats (not illustrated) were higher (all $P < 0.0001$, paired $t$ test) compared with the iron-restricted groups.

**FIGURE 2**  Plasma iron (A), plasma percentage of transferrin saturation (%TS) (B), and liver iron (C) after the development of iron deficiency, marginal vitamin A (VA) deficiency and their combination. Data are expressed as the mean ± SEM, $n = 6$ /group except for the $^{-}$Fe VA group, $n = 5$. Means without a common letter differ, $P < 0.05$. Boxes display the results of 2-way ANOVA, conducted on log (ln)-transformed data for plasma iron. By post-hoc analysis (Fisher’s Protected Least Significant Difference test) after 2-way ANOVA, VA was significant, $P = 0.0376$ for plasma iron and $P = 0.0416$ for %TS. Abbreviations: $^{+}$Fe, iron adequate; $^{-}$Fe, iron deficient; $^{+}$VA, VA adequate; VAm, VA marginal. Values for weight-matched rats (not illustrated) were higher (all $P < 0.01$, paired $t$ test) compared with the iron-restricted groups.
Marginal VA deficiency slightly attenuated the increase in transferrin protein expression due to iron deficiency because the FêVA and FeVAm groups tended to differ ($P = 0.059$, t-test).

Transferrin receptor protein expression in liver homogenates was also analyzed by ELISA (Table 4). Rats fed either of the iron-restricted diets had elevated levels of transferrin receptor protein ($P = 0.0001$, 2-way ANOVA), as previously reported (41), but there was no effect of dietary VA ($P = 0.55$) and no interaction ($P = 0.70$).

Liver ferritin heavy-chain mRNA expression did not differ among the four diet treatments (Table 4). In contrast, there was an effect of iron, but not of VA, on liver ferritin light-chain mRNA (Fig. 5A). Expression was significantly increased in the FeVAm group, with an intermediate increase in the FêVA group. Ferritin light-chain protein expression (Fig. 5B) followed a pattern similar to ferritin light-chain mRNA. There were no significant differences in hemopexin or CRBP mRNA among the four treatment groups (Table 4). Cp mRNA was lower in the FeVAm group.

**DISCUSSION**

We evaluated the effects of marginal VA deficiency, iron deficiency and their combination on growth, feed efficiency, and plasma and tissue indices of iron and VA status during the period of rapid postweaning growth in rats. The experimental design was selected to represent, in an animal model, conditions that are prevalent in many humans populations from childhood through adolescence. Among young children in developing countries, it is common for iron deficiency to be sufficiently severe to limit the child’s rate of growth, as indicated by improved growth after iron supplementation (42). In contrast, VA supplementation has rarely improved growth, suggesting that most VA deficiencies in children are “mild” with respect to this functional outcome (43–46). The degree of iron deficiency produced by our iron-restricted diets was severe enough to impair weight gain after 2 wk and to reduce final body weights by nearly half (Table 2). The degree of VA deficiency was chosen to be marginal because we contend that this scenario provides a good model for the VA status most
common in young children in developing countries. The levels of iron and VA in the control diet were chosen to meet requirements but were below the NRC recommendations (47), which are set to include a margin of safety. Nonetheless, there were significant differences in several of the indices of iron and VA status that we measured when dietary iron, VA, or both, were below the NRC recommendations (47), but were below the NRC recommendations (47), which are set to include a margin of safety. Nonetheless, there were significant differences in several of the indices of iron and VA status that we measured when dietary iron, VA, or both, were further reduced.

Iron deficiency results not only in reduced food intake but also in reduced feed efficiency [(29,48) and Table 2]. To address the possible confounding of growth restriction on iron deficiency (29), we included, in addition to the four groups comprising the main 2 × 2 study, two parallel groups of WM rats for direct comparisons to the iron-restricted groups. Although weight gain has been used previously as a statistical covariate to control for the effects of iron deficiency on growth (30), we reasoned that congruence between this statistical approach and a direct comparison would provide additional assurance that growth impairment was not a confounding cause for the observed effects of dietary micronutrient restriction on the indices measured. Because we anticipated there would be little, if any, effect of marginal VA deficiency on growth within the time frame of this experiment (and none was observed, Table 2), we did not include WM controls for this group. Due to differences in the efficiency of feed utilization (Table 2), it was necessary to feed ~10% less of the micronutrient-adequate diet to the WM rats, compared with the amount of Fe diet consumed spontaneously by iron-restricted rats, to maintain equal body weights throughout the 5-wk study. Weight matching is not strictly a control for either energy intake or micronutrient intake. Although the WM rats were micronutrient adequate, they should not be (and were not) compared with the control (Fe^−VA) rats because their micronutrient intakes were not identical. Despite the limitations, the inclusion of the WM rats is an important aspect of our design because it helps to ensure that growth restriction per se is not an explanation for most of the observed effects of iron restriction.

Blood iron variables are among those most often used to assess iron status. Both Hb and Ht decreased within 2 wk after commencement of dietary treatment in the iron-restricted groups. The effects of VA deficiency on Hb and Ht have varied in previous studies. In some rat studies, blood Hb and Ht declined during VA deficiency (23,25,30), whereas in others, they did not differ from control levels (14,24). In our 5-wk study, the induction of marginal VA deficiency did not affect blood Hb or Ht. It is possible that the severity of the iron deficiency induced in our study masked any effect of marginal VA deficiency on these indicators of iron status, or that marginal VA deficiency is not by itself a risk factor for low Hb and Ht.

The concentrations of plasma and liver iron in rats fed the Fe diets were within the range expected, whereas those for rats fed the control level of iron (20–22 µg/g diet) were somewhat lower than in iron-sufficient rats in a previous study (28). Although an increase in liver iron stores during VA deficiency has been noted previously (6,21,22,35,49), we and others (24) did not observe an elevation in liver iron in marginal VA deficiency, even when spleen and bone marrow iron stores were increased (24). The elevation in liver iron concentration reported by Mejia et al. (21) occurred only in

### TABLE 4

<table>
<thead>
<tr>
<th>Diet</th>
<th>Tfr Protein mRNA</th>
<th>Hemopexin mRNA</th>
<th>CRBP mRNA</th>
<th>Cp mRNA</th>
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<tr>
<td>^Fe^+VA</td>
<td>1.00 ± 0.13^a</td>
<td>1.00 ± 0.08</td>
<td>1.00 ± 0.11</td>
<td>1.00 ± 0.06^b</td>
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<tr>
<td>^Fe^+VA_m</td>
<td>1.03 ± 0.18^a</td>
<td>1.00 ± 0.07</td>
<td>1.00 ± 0.17</td>
<td>1.04 ± 0.15</td>
</tr>
<tr>
<td>^Fe^−VA</td>
<td>1.68 ± 0.09^b</td>
<td>1.07 ± 0.64</td>
<td>0.89 ± 0.06</td>
<td>0.78 ± 0.11</td>
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<tr>
<td>^Fe^−VA_m</td>
<td>1.82 ± 0.07^b</td>
<td>1.08 ± 0.05</td>
<td>0.78 ± 0.06</td>
<td>0.89 ± 0.04</td>
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</tbody>
</table>

1 Values are means ± SEM, n = 6. Means in a column without a common letter differ, P < 0.05. Abbreviations: Cp, ceruloplasmin; CRBP, cellular retinol-binding protein. Values for weight-matched rats (not shown) were lower, compared to their respective iron-restricted groups, for Tfr protein (P < 0.03), but did not differ for Cp.

**FIGURE 5** Ferritin light-chain mRNA (A) and protein (B) in liver of rats after the development of iron deficiency, marginal vitamin A (VA) deficiency and their combination. A. For ferritin light-chain mRNA, n = 6 except for the ^Fe^−VA group, n = 5. For ferritin light-chain protein, n = 3 pools (one pool per block). Data are expressed as the mean ± SEM. Means without a common letter differ, P < 0.05. Boxes display the results of 2-way ANOVA. Abbreviations: ^Fe^, iron adequate; ^Fe^−, iron deficient; ^VA^, VA adequate; ^VA_m^, VA marginal. Values for weight-matched (WM) rats (not illustrated) were lower for ferritin light-chain mRNA (all P < 0.002, paired t test) compared with their respective iron-restricted groups. Values for ferritin light-chain protein were lower in WM rats vs. ^Fe^VA_m, rats (P = 0.03), but did not differ between the WM and ^Fe^−VA groups.
adult rats after 50 d of consuming a VA-deficient diet. It is possible that our study was not long enough for liver iron stores to become significantly elevated. Together, these data imply that the effects of VA deficiency are dose and/or time dependent, and that some indicators of iron status may be more responsive than others to a marginal deficiency of VA.

We hypothesized that the genes for transferrin, ferritin light chain, ferritin heavy chain and ceruloplasmin were all good iron-related candidates for regulation, whose expression could also be affected by VA status. Transferrin receptor expression is sensitive to the iron status of an individual and the translational control involved in its sensitivity to iron status is well characterized [reviewed in (50,51)]. The transferrin receptor gene may also be controlled transcriptionally, although this is less well characterized, and may also be affected by VA status (52) and retinoic acid (53). Before conducting the main study, we had conducted a pilot study in VA-deficient rats that suggested that transferrin mRNA might be reduced by VA deficiency. In our main study, iron deficiency, as previously documented (54), increased the level of transferrin mRNA and protein (Fig. 4), which may be a mechanism to maintain the delivery of iron to tissues. Although marginal VA deficiency was not a significant factor, the reduction in transferrin mRNA in rats with more severe VA deficiency (5) suggests that VA is a factor under conditions of more severe or prolonged deficiency.

As expected, iron deficiency also increased the level of liver transferrin receptor protein due to the well-characterized post-transcriptional stabilization of transferrin receptor mRNA by iron regulatory proteins (IRP) during iron deficiency (55). Marginal VA deficiency had no effect; however, it is possible that, as for transferrin mRNA in our pilot study, transferrin receptor expression is regulated when VA deficiency is more severe. Ferritin light and ferritin heavy chains have been described as having functionally different roles (56,57). Ferritin light chain confers stability to the ferritin apoprotein and appears to play a role in long-term storage of iron within the ferritin protein complex. Ferritin heavy chain appears to play a greater role in mobilization of iron to and from the ferritin protein complex. Ferritin heavy chain is responsible for, or capable of, more rapid iron uptake by iron-deficient hepatic and erythroid lineage cells (64,65). VA status and retinoids have also been shown to influence serum Cp levels (66). Moreover, Cp is a positive acute phase protein whose concentration is elevated in plasma during inflammation and infection. However, Cp mRNA was not elevated in any of the treatment groups in our study. The second gene related to the acute phase response was hemopexin, which protects cells from heme-mediated oxidative damage by binding to heme, decreasing its peroxidative activity and transporting it via endocytosis into cells expressing hemopexin receptors (67). Hemopexin mRNA also did not differ with VA status. The lack of an elevation in hemopexin as well as ceruloplasmin implies that the rats in our study were not in an inflammatory state.

Indices of VA status were affected by marginal VA deficiency, as expected, and some were also affected by iron deficiency. Plasma retinol was reduced in rats with marginal VA deficiency, regardless of iron status, and by iron deficiency alone. Interestingly, the concentration of plasma retinol was lowest in rats with combined iron and marginal VA deficiency (Fig. 3). Plasma retinol was somewhat lower than expected in the control group, possibly due to lower preweaning liver stores (68) compared with most weanling rats that have been nursed by dams fed a stock diet, and to the lower VA concentration of our control diet, as discussed above. Despite this, the effects of marginal VA deficiency and iron deficiency on plasma retinol were significant.

Like plasma retinol, liver total VA (both retinol concentration and contents) was significantly affected by dietary VA, iron and the combination. Iron deficiency increased in liver retinol which, taken together with low plasma retinol, suggests
that iron deficiency promotes the sequestration of retinol in the liver. The increase in liver VA in iron deficiency is in agreement with the results from a previous kinetic study in which the mobilization of liver VA was decreased in iron deficiency (69). The elevation of liver VA in iron deficiency and the lower plasma iron and %TS in marginal VA deficiency imply that there is a bidirectional interaction between VA and iron mobilization from storage. Collectively, these results suggest a greater complexity in the metabolism of these micronutrients than previously appreciated.

Cellular retinol-binding protein I (CRBP-I) mRNA was also analyzed in liver because it has been shown to be somewhat reduced in the liver of rats fed a VA-free diet (70), and has been reported to be higher in cells grown under iron-deficient conditions (71). Because CRBP-I controls the concentration of free retinol by binding retinol within cells, an increase in CRBP-I mRNA in the liver could play a role in the increased liver retinol storage and decreased plasma retinol concentrations we observed in the Fe−VA group (Fig. 3). However, CRBP-I mRNA did not differ among treatment groups (Table 4). This result also suggests that the VA deficiency status of our rats was indeed marginal, neither severe enough nor prolonged enough to affect CRBP-I at the mRNA level.

In summary, in the context of adequate dietary iron, marginal VA deficiency had no effect on Hb, Ht or liver iron. There were, however, main effects of dietary VA on plasma iron and %TS, as supported by post-hoc analysis (Fig 2A, B). In the context of relatively severe iron deficiency, marginal VA deficiency did not exacerbate any of the indices of iron status that we measured. Nevertheless we contend that the trends observed with marginal VA deficiency alone support the importance of adequate VA for normal iron metabolism, especially as related to plasma iron and transferrin saturation. Overall, the results of this study lend support to the hypothesis that marginal VA deficiency may hamper the effectiveness of an iron intervention program, and that supplementation or fortification with combinations of iron and VA may be more likely than treatment with iron alone to improve the iron and VA status of human populations in which these deficiency conditions coexist.

LITERATURE CITED


