Effects of iron deficiency upon the antibody response to influenza virus in rats

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The effects of severe and moderate iron deficiency upon the antibody response to influenza virus were investigated in rats. Three groups of weanling male Wistar rats were fed one of two iron-deficient diets (5 mg and 15 mg iron/kg diet) or a normal iron-containing diet (35 mg iron/kg diet). A group of individually pair-fed rats was introduced with the low iron-consuming rats. The effects of the diets upon various iron status parameters were followed during the 4th, 5th, 6th, and 7th week of diet. After 4 weeks of feeding different diets, an intraperitoneal injection of inactivated influenza virus A/New Jersey/76 was performed and a recall injection was done at 5 weeks. Primary and secondary antibody responses were assayed. Rats were sacrificed at 7 weeks of diet. After 4 weeks of feeding different diets, the rats fed the 5 mg iron/kg diet were severely anemic and rats fed 15 mg iron/kg diet were moderately iron-deficient, as shown by their iron status parameters. Growth was delayed in anemic and matched pair-fed rats. A primary antibody response was almost nonexistent in all groups. Secondary antibody titers were significantly weaker in anemic rats than in ad libitum controls, but were not different from those of pair-fed rats. This response was similar in moderately iron-deficient, ad libitum, and pair-fed rats. These results show that antibody synthesis in response to the influenza virus vaccine is preserved in moderate iron deficiency but is reduced in severe anemia. The reduction in energy consumption associated with severe iron deficiency in the rat could play a part in the altered humoral response.

Keywords: iron deficiency; influenza virus; vaccination; rat

Introduction

The efficiency of vaccination in humans is of great importance in subsequent resistance to infections. Bearing in mind the high prevalence of iron deficiency both in industrialized and in developing countries,[1-5] it was of interest to further investigate the effects of severe and moderate iron deficiency upon antibody formation following vaccination.

Indeed, several important metabolic functions are altered, even in cases of moderate iron deficiency, prior to depletion of iron stores. In particular, iron-containing enzymes or those enzymes requiring iron as a co-factor have been shown to be depressed in several organs.[6-8] These results have provided a possible explanation for the deleterious consequences of iron deficiency on growth rate, physical work capacity, behavior, pregnancy, and resistance to infections.[9,10]

The effects of iron deficiency upon cellular immunity and monocyte/macrophage functions have been more extensively studied in humans and animals than has humoral immunity (see review[11]). Results on the former have met with the agreement of most authors. For cellular immunity in humans, a decrease was observed in the number of circulating T lymphocytes and in their blastogenic response towards several mitogens and antigens, as well as a correction of these anomalies after iron supplementation.[12-17] The decrease in tissue iron stores rather than anemia appeared to be an important factor in impairment of cellular immunity.[18,19] Animal studies on mice and rats support ob-

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servations made in human studies in severe and even in moderate iron deficiency.[20-22]

Phagocytosis was rarely shown to decrease in human iron deficiency,[18,23,24] but this was not the case for bactericidal activity against several microorganisms.[18,25-27] Alterations in some polymorphonuclear functions were also found in animal studies, though these were subject to controversy depending on the age of the animals.[28-30]

In humans, humoral immunity is far less affected by iron deficiency than cellular immunity. Antibody titers against tetanus toxoid[17,21,24] were similar in iron-deficient and control children. However, in contrast to human studies, animal studies suggested impairment of humoral immunity: a decreased blastogenic response of B lymphocytes towards LPS in anemic mice[31] and various alterations in basal serum immunoglobulin concentrations in moderately or severely iron-deficient animals.[21,32] Only the response to immunization with tetanus toxoid was studied in rats fed with low iron-containing diet. This response was weak in iron-deficient rats and was correlated with the decrease in the iron content of the diet: decreased response occurred even with 10% decrease in the dietary iron content.[32]

The present study was designed to explore the effects of severe and moderate iron deficiency in rats upon primary and secondary antibody responses towards inactivated porcine influenza vaccine containing virus A/New Jersey/76.

Materials and methods

Animals and diets

Weanling male Wistar rats (Charles Rivers, St Aubin-les-Elboeufs, France) aged 21 days, wt 47 to 61 g, were housed individually in plastic cages with stainless steel wire mesh bottoms under a fixed light-dark cycle (lights on from 8:00 to 20:00), constant humidity, and temperature (22°C). All materials in contact with rats or diet preparations were of stainless steel, glass, or plastic in order to avoid iron contamination. Rats were randomly assigned to four dietary treatment groups of 9 to 10 rats upon arrival in the laboratory. Diets were of low, medium, or normal iron content. Group 1 (low iron-containing diet) was fed a commercial iron-deficient diet (UAR, Villemoisson-sur-Orge; France) containing 4 to 5 mg Fe/kg diet (powder). The composition of the diet is shown in *Table 1*. The diet was adequate in all nutrients except iron; in particular, the levels of zinc (roughly 40 mg/kg diet) and selenium (0.77 mg/kg diet) allowed normal growth and development of the animals.[8,33] Group 2 (medium iron-containing diet) and the control group were fed the basal iron-deficient diet to which ferrous sulfate was added ($FeSO_4$,7 H_2O) so that final concentrations were, respectively, 15 and 35 mg Fe/kg diet.

Different groups were fed their respective diets and received distilled water ad libitum for 7 weeks. The control group (35 mg Fe/kg diet) was given the recommended dietary iron level.[34] The fourth group (pair-fed controls) also received the normal iron-containing diet; individual pair-feeding was done on a two-day basis after accounting for food spillage.

Table 1 Composition of the basal iron-deficient diet

Ingredient	Percentage by weight
Spray dried milk	65.0
Sucrose	21.1
Lard	6.6
Peanut oil	3.3
Mineral and vitamin mixture[a]	4.0

[a] Composition of the mineral and vitamin mixture in the diet (mg/kg diet): retinyl acetate, 0.016; thiamin, 0.01; riboflavin, 0.005; calcium pantothenate, 0.012; pyridoxine, 0.005; mesoinositol, 0.2; vitamin B12, 0.015; ergocalciferaol, 0.005; α-tocopheryl acetate, 0.6; menadione, 0.004; niacin, 0.01; folic acid, 0.001; choline, 4; biotin, 0.01; p-aminobenzoic acid, 0.01; sodium chloride, 6.005; potassium iodide, 0.00095; manganous sulfate, 0.27; copper sulfate, 0.08; excipient (sucrose), 28.74105.

Animals were weighed upon arrival in the laboratory and weekly thereafter.

Vaccination

At the 4th week of feeding different diets and after slight anesthesia with ether, approximately 500 μl of free-flowing blood from the tail vein were taken, for hemogram determination (hemoglobin, hematocrit, red blood cell count, mean corpuscular volume, and erythrocyte protoporphyrin) and for determination of basal antibody titers. Serum was obtained after centrifugation the subsequent day, and antibody titers were determined by the hemagglutination inhibition.[35]

Following 4 weeks of the feeding period, all rats received an intraperitoneal injection of inactivated porcine influenza vaccine A/New Jersey/76, approximately 1 μg of purified hemagglutinin, representing one tenth of a human dose. This vaccine strain has been chosen because this virus is less sensitive to non-specific hemagglutination inhibitors, and antibodies are therefore easier to measure in rat serum. During the 5th week, a second blood sample was taken for evaluation of iron status and primary antibody response, and sensitized rats received a booster injection of the same dose of vaccine. After 6 and 7 weeks of feeding, blood samples were drawn again and used for evaluation of iron status, and for secondary antibody response.

Evaluation of iron status

After 4 weeks of feeding different diets and each week thereafter for the following 3 weeks, measurements of hemoglobin, hematocrit, and red blood cell parameters were performed on a Coulter Counter S560 (Coultronics France, Margency, France); erythrocyte protoporphyrin was measured on a hematofluorometer (ZPPMetter Aviv, Analis, Namur, Belgium).

At the end of the feeding period, rats were anesthetized lethally with ether. The liver was then excised

and used for iron concentration and total iron content by dry ashing (method of Schricker et al.[36]) and atomic absorption spectrophotometry (Pye Unicam SP9, Phillips, France).

Statistical analyses

For biological variables, differences between groups were tested by standard one-way analysis of variance (ANOVA) or Student's *t* test. The non-parametric test of Mann and Whitney (U test) was used for antibody titer comparisons between groups.

Results

Evaluation of iron status

The evolution of the hemoglobin level (Hb), hematocrit (Ht), mean corpuscular volume (MCV), and erythrocyte protoporphyrin (EP) is shown in *Figure 1*. For all parameters except Hb and Ht levels, and regardless of the week the measurements were made (4th, 5th, 6th, or 7th week), there were no significant differences between ad libitum control rats and pair-fed controls. Following 5 weeks of the feeding period and up until the end of the study, Hb levels were significantly higher in pair-fed rats than in controls ($P < 0.01$ at the 5th and 6th weeks; $P < 0.02$ at the 7th week). The Ht levels were also significantly higher in pair-fed animals than in controls at the 6th and 7th weeks ($P < 0.05$).

There was a highly significant difference following 4 weeks of the feeding period (and until the end of the experiment) between the mean Hb level of moderately or severely iron-deficient rats and that of controls or pair-fed animals ($P < 0.001$). The Hb level was also

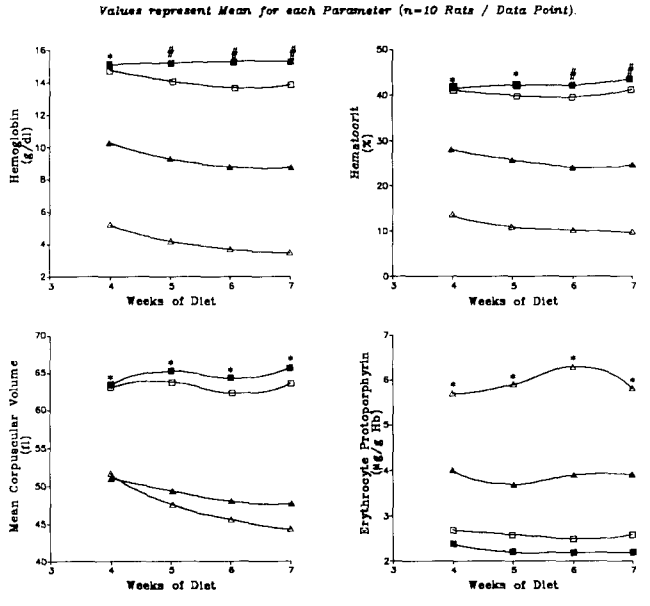


Figure 1 Evolution of the hemoglobin, hematocrit, mean corpuscular volume and erythrocyte protoporphyrin levels.

Table 2 Liver weights, liver iron concentration, and total liver iron in severely and moderately iron-deficient rats, pair-fed, and normal rats[a,b]

Treatment group (mg iron/kg diet)	Liver weight (g)	Liver iron concentration (µg/g fresh liver)	Total liver iron (µg)
5	5.8 ± 0.6[a]	26.4 ± 1.1[a]	157.7 ± 23.2[a]
15	9.6 ± 0.4[b]	39.3 ± 0.9[b]	378.0 ± 19.4[b]
35 (PF)[c]	6.8 ± 0.3[a]	73.8 ± 4.0[c]	504.1 ± 42.4[c]
35 (C)[c]	11.6 ± 0.4[c]	49.2 ± 3.5[d]	580.6 ± 59.2[c]

[a] Values are means ± SEM for ten animals. Comparisons of all four groups: $P < 0.01$ for each parameter.
[b] Means not sharing a common superscript letter are significantly different at $P < 0.02$.
[c] PF: pair-fed. C: controls.

statistically lower in the group fed the low iron-containing diet than in the group fed the medium iron-containing diet ($P < 0.001$). The same was observed for Ht level and MCV. However, differences in MCV between rats fed 5 or 15 mg iron/kg diet were observed only after 5 weeks of feeding ($P < 0.05$) and increased during the 7 weeks of the feeding period ($P < 0.01$ at the end).

EP increased in the iron-deficient groups compared to controls and pair-fed rats after 4 weeks of feeding ($P < 0.001$) and was higher in the group fed the low iron-containing diet than in the group fed the medium iron-containing diet ($P < 0.001$). The differences remained similar until the end of the feeding schedule.

These results led us to consider the group which was fed 5 mg iron/kg diet as being severely iron-deficient (mean Hb < 5 g/dl), and the group fed 15 mg iron/kg diet as moderately iron-deficient (mean Hb < 11g/dl and EP < 3 µg/g Hb). They confirmed that ad libitum and pair-fed controls had normal iron status. When iron status indicators were compared between the 4th, 5th, 6th, and 7th week in each group, no significant modification in any parameters were observed in controls, pair-fed, moderately, or severely iron-deficient rats.

The mean liver weights, liver iron concentration, and total liver iron are shown in *Table 2*. When all groups were considered, liver weight and body weight were significantly correlated after 3 weeks of diet and up until the end of the study: r = 0.63 at the 3rd week ($P < 0.01$), and r = 0.95 at the 7th week ($P < 10^{-9}$).

The mean liver weight of anemic rats was similar to the liver weight of pair-fed rats. Both were lower than that of controls and moderately iron-deficient rats ($P < 0.001$). Liver weight of moderately iron-deficient animals was also lower than that of control rats ($P < 0.01$).

The liver iron concentration was lower in ad libitum controls than in pair-fed controls ($P < 0.001$), but although liver weight was higher in the former, total liver iron was similar in pair-fed and control rats. The liver iron concentration was significantly lower in anemic and moderately iron-deficient rats than in ad libi-

tum controls ($P < 0.001$ and $P < 0.02$, respectively) and pair-fed controls ($P < 0.001$). Differences were also significant between anemic and moderately iron-deficient rats ($P < 0.02$). The same results were observed for total liver iron stores.

Effects of iron deficiency on growth

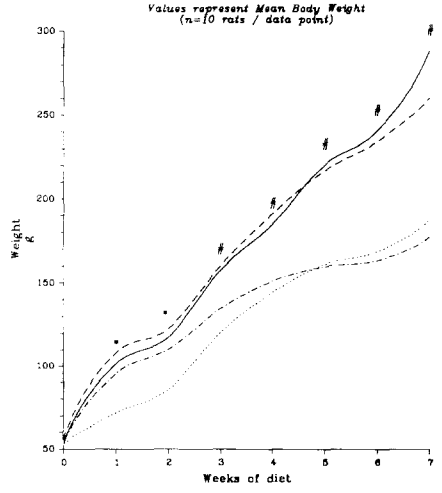
The evolution in body weight during the experiment is shown in *Figure 2*. Mean body weights did not differ at the beginning of the experiment. After one week of the respective diets, a significant difference in body weight appeared between pair-fed rats and the other three groups. The differences in body weight of pair-fed rats and controls or moderately iron-deficient animals increased until the end of the feeding schedule ($P < 0.001$).

Statistically significant differences between mean body weights of anemic rats and controls or moderately iron-deficient rats appeared after 3 weeks of diets and up until the 7th week of diet ($P < 0.001$). Conversely, mean body weights of anemic and pair-fed rats were no different after 3 weeks of diet and up until the end of the experiment. Moderately iron-deficient rats were not different from controls except at the last week of diet ($P < 0.01$).

Effect of iron deficiency on antibody response to attenuated porcine influenza vaccine

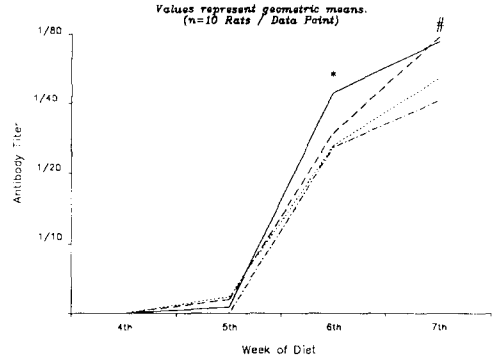
The evolution of log antibody titers in each group is shown in *Figure 3*. None of the rats expressed a basal antibody titer after 4 weeks of feeding different diets, and prior to the first sensitization, indicating that they had not been previously in contact with the virus.

After the first injection, the primary antibody response was very weak in the four groups (one response out of 10 in ad libitum controls, 2 out of 10 in moderately iron-deficient rats, none in anemic rats,



* *Significantly different Values between pair-fed Rats (····) and the three other groups: Normal Iron-consuming Rats (——), medium Iron-consuming Rats (– –) and low Iron-consumming Rats (–·–), p ⟨ 0.01.*
\# *Significantly different values between low Iron-consumming rats and normal or medium Iron-consumming Rats (p ⟨ 0.01), with no significative difference between low Iron-consumming Rats and pair-fed Rats.*

Figure 2 Evolution of weight.



* *Significantly different Values between low (–·–) and normal (——) Iron-consuming Rats, p ⟨ 0.05.*
\# *Significantly different values between low Iron-consuming Rats (–·–) and normal (——) or medium (– –) Iron-consuming Rats, p ⟨ 0.05.*
(····) *Pair-Fed.*

Figure 3 Evolution of antibody titer.

and 1 out of 10 in pair-fed rats). One week after the second immunization of sensitized animals, the antibody titers were significantly lower in anemic rats than in ad libitum controls ($P < 0.05$), but differences were not significant among the other groups. The secondary antibody response, measured 15 days after the second immunization, was also similar in ad libitum controls, pair-fed, and moderately iron-deficient rats, but remained significantly lower in anemic rats than in controls and moderately iron-deficient animals ($P < 0.05$). It was not different between anemic and pair-fed rats.

Discussion

The decrease in body weight of severely anemic rats after a three-week ad libitum intake of an iron-deficient diet and up until the seventh week of diet is consistent with the finding of other investigators[37-41] and with our previous studies.[8] However, the pair-fed control group included in this study enabled us to evaluate the respective roles of iron deficiency and decreased food consumption upon growth rate in anemic rats. The absence of significant differences in the mean body weight of anemic and pair-fed rats and the growth rate following 3 weeks of feeding and up until the seventh week, indicated that anorexia and limited energy intake, rather than anemia per se, decreased the body weight and growth rate. Growth rate was reduced in pair-fed animals after only one week of the feeding period. But other investigators have found a significant reduction in growth rate and mean body weights in mice only after 3 weeks of feeding.[20]

The iron status of the pair-fed group was identical to that of control rats, in agreement with a previous study we had performed. This observation clearly showed that, in rats, food restriction had no effect upon iron status parameters. This might be due to an increase in iron absorption in order to compensate for the low iron intake. The higher hemoglobin and hematocrit levels, along with the higher liver iron concentration than ad libitum controls, are in favor of this hypothesis. It is noteworthy that, despite the lower liver weight of pair-fed rats compared to ad libitum con-

trols, the slight increase in liver iron concentrations in the former led to similar total hepatic iron stores in both groups.

No studies have been performed in humans or animals concerning the effects of iron deficiency upon antibody production in response to the vaccination against influenza virus. Two studies measured antibody titers after vaccination against tetanus toxoid in iron-deficient children[24]: both primary and secondary antibody titers were similar in iron-deficient and control children. In another study,[42] the primary antibody response of iron-deficient children to diphtheria and typhoid immunization was measured: in 5 children immunized with diphtheria toxoid, there was no increase in antitoxin; of 8 children who were given typhoid vaccine, 6 responded by an increase in Salmonelle typhi O agglutinin titer. However, not enough studies have been performed, nor patients tested, to be able to draw any conclusions. Conversely, animal studies have suggested an impairment of humoral immunity. Serum concentrations of immunoglobulins M and G were either increased or decreased in iron deficiency depending on the murine species tested (mice or rats, see review[11]). The number of plaque-forming cells was reduced in iron-deficient adult rats and mice after immunization with sheep red cells.[28,31] The response to immunization with tetanus toxoid was weak in iron-deficient rats and was correlated with a decrease in the iron-content of the diet.[32]

A/New Jersey/76 is a T-dependent antigen.[43] Humoral responses towards immunization are therefore dependent upon cellular immunity, through cooperation with T helper and T suppressor cells. In the present study, the decreased antibody titers in anemic rats could be explained by a defect in T helpers or T suppressors and/or in lymphokines connecting T and B lymphocytes. The observation that pair-fed rats were no different from anemic rats could indicate that the restriction in energy intake rather than anemia was responsible for the low antibody response. Low food intake could decrease cellular protein synthesis, especially that of T cell lymphokines and B cell immunoglobulins. However, iron deficiency may have a specific deleterious effect upon the number and/or function of some T lymphocyte subsets. Comparisons with ad libitum controls were also significant with anemic rats, but were not statistically significant with pair-fed animals, suggesting that in severely iron-deficient rats, the above two mechanisms could act synergistically to decrease antibody response. This result may be of special relevance in cases of human anemia in developing countries, which couple iron deficiency anemia with concomitant protein-energy malnutrition. Some studies have shown that lymphocyte memory is not altered in iron deficiency. Rather, transformation or effector mechanisms may be affected.[44]

Moderate iron deficiency, sufficient for significantly decreasing iron status parameters and iron stores, had no effect upon antibody response to vaccination. No studies have been performed so far on the antibody response after vaccination of moderately iron-deficient animals, but circulating immunoglobulin levels have been investigated during moderate and severe iron deficiency in rats and mice.[21,32] Some differences between mice and rats were observed depending on the severity of iron deficiency and type of immunoglobulin. More studies concerning the effects of several degrees of iron deficiency upon the proportion of T lymphocyte subsets in certain organs and the mechanisms at the cellular level (expression of transferrin receptors, lymphokines, and cellular cooperation factor synthesis) are needed in order to understand the different alterations observed in our and other experimental studies. A pair-fed control group appears to be necessary in all animal studies on iron deficiency, taking into account the possible confusing effect between anemia and decreased food consumption.

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