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Maternal and Neonatal Tissue Lysozyme Levels: Effects of Iron Nutriture in Rats (40684)

IRA WOLINSKY* AND ADRIA ROTHMAN SHERMAN†

*Department of Human Development and Consumer Sciences, University of Houston, Houston, Texas 77004, and †Department of Foods and Nutrition, University of Illinois, Urbana, Illinois 61801

Iron deficiency anemia is a common nutritional problem among females of childbearing age and children (1). In a continuing series of experiments in our laboratories, we have investigated the effects of maternal iron deficiency on metabolism in the offspring (2–5). Maternal iron restriction during pregnancy and lactation results, in the offspring, in numerous metabolic alterations including hyperlipidemia (2, 4), apparently related to increased endogenous triglyceride production (4), and a decreased ratio of Zn/Cu in some tissues (3, 5).

Iron deficiency in the adult female rat has been shown to result in increased kidney lysozyme (EC 3.2.1.17) activity when compared with control animals (6). Lysozyme is a bacteriolytic enzyme discovered by Sir A. Fleming in 1922 (7) and has been the focus of considerable interest for more than half a century (8). It is considered to be one of several nonspecific immune mechanisms involved in host defenses (9–11) and is widely distributed in vertebrate tissues (12, 13). Modified levels of lysozyme have been reported in a wide range of pathological conditions and stimuli (8, 14–17) and nutritional states.

In animal studies, lysozyme content of various tissues has been reported to increase during vitamin A deficiency (18) and hypervitaminosis D (19). Lysozyme was found in significantly reduced concentration in tears of malnourished preschool-age children, along with reductions in secretory IgA and amylase (20). In another study of children Chandra *et al.* (21) observed that noninfected malnourished children had lower intracellular and plasma lysozyme activity than healthy controls.

Generally, the interrelationships between nutritional status and lysozyme action merit further study. Our observation of altered kidney lysozyme levels during iron deficiency (6) led us to speculate that the iron-deficient organism, with a lower immune response (22, 23), may require more lysozyme for its antibacterial functions.

In the investigation reported here, we studied the effects of iron restriction on tissue lysozyme levels in maternal rats and their suckling offspring and present previously unreported age-related differences in tissue lysozyme activity.

Materials and methods. Female, nulliparous, Sprague–Dawley CD rats were bred and placed on experimental diets and glass-distilled water, both *ad libitum*, when they weighed approximately 220 g. The iron-deficient diet provided 5 ppm iron and the control 307 ppm iron (Table I). On the second day of lactation, litters were standardized to contain three female and three male pups. The litters were suckled by their dams which remained with them until the 18th day of lactation when both dams and pups were fasted for 4 hr and killed. Thus, the dams were provided experimental diets for a total of 40 days. Sera, kidneys, and spleens were collected from the dams and pups and frozen for future analysis. Milk was collected on Day 18 by palpation when the dams were under Nembutal anesthesia after administration of oxytocin and was also frozen until analyzed. Lysozyme activity was found to be stable for at least 1 month when tissues were stored at −20°. All tissues were assayed within that time. For analysis of lysozyme, 150 mg of spleen and 150–250 mg of kidney were homogenized in 5 ml (spleen) or 10 ml (kidney) of cold 0.066 *M* potassium phosphate buffer, pH 6.24, and centrifuged at 100,000*g* for 1 hr at 2°. In the case of pup tissues, two organs from each litter were pooled for homogenization and subsequent analysis. After centrifugation, the supernatant was used for measurement of lysozyme activity by monitoring the lysis of cell walls of *Micrococcus lysodeikticus* (24).

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Lysozyme rate was expressed as

$$\text{units/mg protein} = \frac{\Delta A\ 450/\text{min}}{0.001 \times \text{mg protein/reaction mixture}}.$$

Serum was used directly for enzyme analysis. The volumes of supernatant and sera used for the assays were within the linear range of enzyme activity. Protein was measured by the method of Lowry *et al.* (25) with bovine serum albumin as standard. Milk was prepared for assay by precipitating 200 μl with 200 μl of 10% TCA and centrifuging; the supernatant was then dialyzed with water overnight at 4°. The samples were lyophilized, reconstituted with 25 μl H_2O, and applied to a lysoplate with *Micrococcus lysodeikticus* agar. The lytic activity of these extracts was measured quantitatively by the radial diffusion-in-gel technique and is expressed as egg lysozyme equivalents as described by Kuettner *et al.* (26). Hemoglobin was determined on tail blood samples by the method of Crosby *et al.* (27), and microhematocrits as described by Wintrobe (28). Statistical significance between group means was detected using Student's *t* test and that between means of dams and their pups by the paired *t* test (29).

TABLE I. COMPOSITION OF DIETS

	Concentration (%)	
	307 ppm Iron	5 ppm Iron
Casein[a]	22.00	22.00
Sucrose	29.70	29.76
Cornstarch	29.70	29.76
Iron-free salt mix[b]	5.48	5.48
Vitamin mix[c]	1.00	1.00
Corn oil[d]	10.00	10.00
Cellulose[e]	2.00	2.00

[a] Vitamin-free casein, Teklad, Chagrin Falls, Ohio.

[b] The level of minerals used is at least 12.5% more than U.S. National Research Council recommendations for lactation (30). Composition of salt mixture (mg/100 g diet): $CaCO_3$, 1680.0; $CoCl_2 \cdot H_2O$, 0.1; $CuSO_4 \cdot 5H_2O$, 9.8; $MgSO_4 \cdot 7H_2O$, 278.5; $MnSO_4 \cdot H_2O$, 19.0; KI, 0.02; NaCl, 1000.0; K_2HPO_4, 2483.6; $ZnCl_2$, 3.0. $FeSO_4 \cdot 7H_2O$ was added to the supplemented diet in place of sucrose and cornstarch.

[c] Teklad, Chagrin Falls, Ohio. Supplies in mg per kg of diet when added at 1% of diet: *p*-aminobenzoic acid, 110.1; ascorbic acid, 991.2; biotin, 0.4; vitamin B_{12}, 0.03; calcium panthothenate, 66.1; choline, 1433.7; folic acid, 2.0; inositol, 110.1; menadione, 49.6; niacin, 99.1; pyridoxine-HCl, 22.0; riboflavin, 22.0; thiamine-HCl, 22.0. Supplies, in units per kg of diet, when added at 1% of diet: vitamin A–palmitate, 19,824; vitamin D_2, 2203; vitamin E acetate, 121. Cornstarch dilutent QS.

[d] Mazola corn oil, Best Foods, Englewood Cliffs, N.J.

[e] Alphacel nonnutritive cellulose, Chagrin Falls, Ohio, with iron extracted by the method of Houk *et al.* (31).

Results. As indicated in Table II, the mean body weights, hemoglobin levels, and hematocrits of iron-deficient pups were significantly lower ($P < 0.001$) than those of control pups. Similarly iron-deficient dams had lower ($P < 0.001$) hemoglobin and hematocrit levels than did control animals.

The growth retardation in iron-deficient pups and maintenance of normal body weights in dams observed in the present study confirm results of previous studies (2–4). The extent of anemia as determined by hemoglobin and hematocrit values reported here for maternal and suckling rat pups is also comparable to our previously reported values (2,

TABLE II. BODY WEIGHTS, WEEKLY FOOD INTAKES, HEMOGLOBIN LEVELS, AND HEMATOCRITS DURING LACTATION

	Food intake (g) during lactation		Body weights (g) during lactation		Day 18 of lactation		
	Week 1	Week 2	Day 8	Day 15	Body wt (g)	Hemoglobin (g/dl)	Hematocrit (%)
Dams							
5 ppm iron	179 ± 21[a]	192 ± 12	312 ± 6	317 ± 5	311 ± 5	12.8 ± 0.5	40.8 ± 0.6
307 ppm iron	154 ± 11	210 ± 26	325 ± 7	328 ± 9	328 ± 9	18.4 ± 0.3	56.0 ± 1.0
	NS[b]	NS	NS	NS	NS	$P < 0.001$	$P < 0.001$
Pups							
5 ppm iron			12 ± 1	23 ± 1	24 ± 2	4.2 ± 0.2	14.2 ± 0.8
307 ppm iron			15 ± 1	32 ± 3	40 ± 5	11.2 ± 1.2	45.8 ± 2.1
			$P < 0.02$	$P < 0.02$	$P < 0.001$	$P < 0.001$	$P < 0.001$

[a] Mean ± SE for eight rats per group.

[b] Statistical significance determined by Student's *t* test.

4). The lower body weights and hematological values of iron-deficient pups are directly related to the iron deficiency per se since maternal food intake and body weights did not differ between groups (Table II). The lower iron concentration of milk which we have reported for iron-deficient dams (4) also contributed to the differences in growth, hemoglobin, and hematocrit levels between groups.

Tissue lysozyme levels (Table III) were found to differ between both dietary groups and between the maternal rats and their offspring. There was an overall trend toward higher tissue lysozyme levels during anemia and substantial differences in lysozyme activity of the different tissues. Renal lysozyme levels were significantly higher in the dams than in the pups for both dietary treatments ($P < 0.005$). However, there were no significant differences in kidney levels of lysozyme between iron-deficient and control dams or pups. Lysozyme levels in the spleen differed with respect to both age and iron nutriture. In the spleen the pups had lysozyme levels approximately twice those found in the lactating adult females ($P < 0.02$). The pups in the iron-deficient group had spleen lysozyme activity which was almost twice as high as that of the pups in the control group ($P < 0.01$). In contrast to this, dams fed both 5 and 307 ppm iron had similar spleen lysozyme levels.

The contribution of spleen lysozyme to the host defense of the suckling pup is thus much greater than in the adult. Effects of iron deficiency on spleen lysozyme were seen only in the pups where it was elevated about twofold in the deficient pups. Thus, we observed significant effects of both age and iron on spleen lysozyme content.

In the control group, serum lysozyme in pups and dams was not significantly different; however, in the pups, it was affected by iron nutriture. A twofold elevation in serum lysozyme was seen in the iron-deficient pups compared to the control pups ($P < 0.001$).

Levels of milk lysozyme (Table III) were determined to test the hypothesis that the iron-deficient dams produced milk with higher lysozyme activity than controls and that this lysozyme was transmitted intact to the sera and/or spleens of the pups. This apparently was not the case since there were no significant differences in milk lysozyme levels as measured on Day 18 of lactation. It is possible that earlier during lactation, possibly during colostrum secretion, (8) when milk lysozyme is highest, there were iron-related differences.

Discussion. Lysozyme no doubt plays an important part in the resistance to infection during perinatal life (33). The age-related differences in tissue lysozyme activity reported in this study point to heretofore unrecognized shifts in the lysozyme levels of different tissues during the life cycle. In the adult, the kidney contributes slightly more than it does in the suckling pup. However, splenic lysozyme is considerably higher in the young than in the mature rat. Serum lysozyme apparently remains constant with age and is sensitive to iron nutriture only in younger animals.

In previous studies in our laboratory, we have also observed histopathologies of the spleen and the thymus in iron-deficient pups[1] which suggest depressed lymphopoiesis in both B- and T-cell areas. It is possible that lysozyme, as a nonspecific factor in immunity, is increased in the iron-deficient organism in an attempt to compensate for the depressed potential for cell-mediated immunological response. The elevation in splenic lysozyme activity in pups may be related to a specific, albeit presently unknown, function of the spleen in lysozyme action during the suckling stage. Thus, the pup's spleen may be more sensitive to the iron deficiency than is the maternal organ.

At this point, knowledge of the physiological function of lysozyme is incomplete, and one can only speculate about the significance of lysozyme changes with iron nutriture (6). Jollés has postulated that in addition to lysozyme's bacteriostatic activity, the peptidoglycans formed by the digestive action of lysozyme on bacterial cell walls may exert an adjuvant or immunostimulating action (11). In the iron-deficient rats with less immunocompetency, this immunostimulating activity of lysozyme may assume greater importance.

[1] Rothenbacher, H., Sherman, A. R. Target organ pathology in iron-deficient suckling rats. (Submitted for publication.)

TABLE III. TISSUE LYSOZYME ACTIVITIES

	Kidney				Spleen				Serum				Milk	
	Dam		Pup		Dam		Pup		Dam		Pup			
	5 ppm Iron	307 ppm Iron	5 ppm Iron	307 ppm Iron	5 ppm Iron	307 ppm Iron	5 ppm Iron	307 ppm Iron	5 ppm Iron	307 ppm Iron	5 ppm Iron	307 ppm Iron	5 ppm Iron	307 ppm Iron
Organ wet weight (g)	1.111[a] ±0.036	1.100 ±0.052	0.187 ±0.014	0.219 ±0.020	0.507 ±0.027	0.537 ±0.036	0.061 ±0.008	0.154*** ±0.021						
Protein (mg/g tissue) or (mg/ml serum)	43.15 ±2.45	45.70 ±2.55	59.74 ±6.85	39.98* ±4.26	59.95 ±3.14	57.47 ±1.98	49.30 ±2.24	45.96 ±1.68	6.81 ±0.12	6.45 ±0.20	7.07 ±0.34	6.59 ±0.19		
Lysozyme (unit/mg protein)	64.52††† ±4.45	59.82††† ±6.03	47.56 ±1.92	44.21 ±4.66	5.43††† ±0.85	5.46† ±0.48	20.56 ±2.51	10.55** ±1.78	3.82 ±0.28	4.25 ±0.25	8.81 ±0.63	4.40*** ±0.53	2.4[b] ±0.6	2.7 ±0.5

[a] Mean ± SE for eight rats per group for kidney, spleen, and milk. Six rats per group for serum.

[b] Milk lysozyme activity expressed as egg white lysozyme equivalents (26).

* Statistical significance between means of iron-deficient and of control pups: $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, determined by Student's t test.

† Statistical significance between dams and their pups in same treatment: $P < 0.02$, †† $P < 0.005$, ††† $P < 0.001$, as determined by paired t test.

It is also possible that in the iron-deficient animal, lactoferrin, an iron-binding protein which like lysozyme is part of the locally secreted mucosal defense system (34), is less effective and lysozyme action increases as a compensation. Possibly the increased level of lysozyme activity in the iron-deficient animal is related to the reported increase in antibacterial action of lysozyme when the chelating agent EDTA is added to *in vitro* cultures (35). Chelating agents bound to trace minerals such as iron may have created an *in vitro* deficient state somewhat comparable to the dietary iron deficiency in our investigations.

Summary. The results of this investigation indicate that iron deficiency in the suckling rats alters immunodevelopment with respect to lysozyme activity. It was also found that suckling and mature rats have different tissue distributions and activities of lysozyme. The health and biomedical implications and significance of these findings are unclear at present.

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