Acta Medica et Biologica
Vol. 42, No. 3, 103-107, 1994

Iron-related Autoimmune Hemolysis: Inductive and Competitive Reactions Found in Lewis Rats

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Received December 22, 1993

Summary. **Iron-related autoimmune disturbances were studied in male amd female Lewis rats. Among 16 males injected with Wister rat liver ferritin, one injected with only the ferritin showed non-hemolytic autoimmune disturbances. A 49 g weight loss, 9.7% siderocytes, high titer anti-nuclear antibody to hepatic nucleus (92%) and low titer auto-red blood cell (RBC) antibody were recognized in this rat. Another male was diagnosed with autoimmune hemolytic anemia after treatment with both the ferritin and anti-Ia monoclonal antibody (mAb) to major histocompatibility complex (MHC) class II, Ia antigen. Auto-RBC antibody was identified in this animal. Mildly or moderately disturbed self-recognitions were found in 5 males treated with ferritin and anti-Ia mAb. On the other hand, all 15 females showed weaker autoimmunity than the males in the same experimental systems. Females differed from males in two points: First, increased iron depositions in proximal convoluted renal tubules were observed in all females injected with the ferritin, especially in one of the females treated together with anti-IA (MHC class I) mAb. Second, atrophic medullae with a relatively hyperplastic cortex were shown in the adrenal glands of 3 females. Among 11 previously pregnant females, 6 had the same atrophic medullae.**

These data suggested the following two conclusions: 1) Disturbed iron incorporation in mitochondria is an important trigger of pre-autoimmune hemolysis in male rats; 2) In female rats, the adrenal and renal tubule axis reacts to improve self-recognition based on immunohemopoietic actions.

INTRODUCTION

Iron uptake in erythroblasts is initiated by transferrin-iron binding to transferrin receptors. Transferrin in the erythroblasts donates most of the iron to mitochondria to incorporate into heme, with the remaining iron to ferritin. When iron is overloaded, the ferritin content of the red cells increases more rapidly than that of liver.[1] Biochemical characteristics of rat liver ferritin have been shown to be different from those of the spleen and intestinal mucosa.[2] Recently, both ferritin L and H subunit mRNAs were found on rat liver membrane-bound polysomes. It was shown that liver ferritin was synthesized on endoplasmic reticulum (ER) membranes.[3] Djeha A et al. reported that iron could affect helper T cell proliferation and suggested the possibility of immunological disturbances associated with iron-overload.[4] It became clear that helper T cell activity was blocked *in vivo*, when the antibody to major histocompatibility complex (MHC) class II antigens was given.[5]

In this paper, antoimmunities due to disturbed iron metabolisms are described. To examine inductive and competitive autoimmune mechanisms, Wister rat liver ferritin was injected into Lewis rats with or without anti-MHC antibodies.

MATERIAL AND METHODS

Animal and experimental protocal

All hosts were Lewis(RT1^l) rats which were maintained in a colony at Hamamatsu University School of Medicine. Six to nine-week-old rats were used as the hosts of experimental numbers (Exp. Nos.) A to E. Initial body weights of male rats were 110 ± 23 g, while those of females were 81 ± 30 g. Wistar rat liver ferritin (UCB, Belgium) was injected once subcutaneously at doses of 3-3.2 μg per rat into 6 males of Exp. No. A and 4 females of Exp. No. B.[2] Wistar rat liver ferritin and mAb to MHC class I antigens were injected once subcutaneously into the groups of 5 males of Exp. No. C and 5 females of Exp. No. D. The

doses of the ferritin and mAb were 3 μg and 4 μg per rat, respectively. The MHC class I mAb, anti-IA mAb, was IgG1 mAb recognizing a monomorphic determinant of rat MHC class I antigens (Sera-Lab, England). Wistar rat liver ferritin and mAb to class II, Ia antigen were injected once subcutaneously at doses of 3 μg and 4 μg, respectively, into 5 males of Exp. No. E. The MHC class II mAb, anti-Ia mAb, was IgG1 mAb recognizing a determinant on the α chain of rat Ia antigen (Sera-Lab, England). In Exp. No. F, 6 females at 7 months of age were inoculated 4 μg anti-Ia mAb subcutaneously once, together with Lewis female spleen cells at a concentration of 0.56×10^8 cells per rat. All these rats were surveilled for 219 to 332 days after immunization.

Hemato-pathological studies

Reticulocytes, siderocytes, Heinz bodies and basophilic erythrocytes were examined in the peripheral blood smears of these rats. Staining procedures used were new methylene blue for reticulocytes, prussian blue reaction for siderocytes, and methyl violet for Heinz bodies. Basophilic erythrocytes were stained with janus green (Chroma, Germany) for 25 min and then counterstaind with Giemsa for 5 min. More than 1,000 erythrocytes were counted to calculate the percentages. The liver, kidney, adrenal gland and spleen of individual specimens were fixed in 20% formalin and embedded in paraffin. Tissue sections were stained with hematoxylin-eosin (H-E). The tissue sections were also stained with prussian blue reaction and counterstained with nuclear fast red. For electron microscopy, small pieces of kidney were fixed in 2% glutaraldehyde, postfixed in 1% osmium tetroxide and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate. They were examined by transmission electron-microscope (TEM).

Immunoglobulin assays

Immuno-histological assay: Hepatic cell smears were prepared from a syngeneic Lewis female (7 m. o.). The sera used in this study were collected when the rats were sacrificed. The smears were incubated with the sera for 45 min and washed in phosphate buffered saline (PBS). Alkaline phosphatase-conjugated anti-

Table 1. Iron metabolisms in Lewis rats injected with rat liver ferritin.

Exp. No.-Rat No.	Host No. (n)	Sex (M, F)	Days after ferritin injection	Reticulocyte % (M±SD)	Basophilic RBC%	Siderocyte % (M±SD)	Iron-deposition: Urinary tubule	Iron-deposition: Hepatocyte
A-1, 4, 5	3	M	308	2.4±0.5	0.4-1.5	2.0±0.2	+	−
A-2*	1	M	236	-	0.7	9.7	+	−
A-6	1	M	229	-	1.3	1.9	+	−
B-2, 3, 4	3	F	332	2.8±1.1	0.8-0.9	1.8±0.8	++	ND
C-1, 2, 4, 5	4	M	250	4.0±0.5	-	1.4±0.1		
C-3	1	M	250	4.4	1.0	5.1		
C-2, 3, 4, 5	4	M	332	2.3±0.2	0.2-1.0	1.6±0.5	+	−
D-2, 3, 4	3	F	331	1.9±0.5	0.4-0.6	1.8±0.1	++	ND
D-5*	1	F	331	3.5	0.5	1.4	+++	+
E-1, 2, 3, 4, 5	5	M	254	1.6±0.5	0.9-1.0	1.6±0.4		
E-1, 5	2	M	325	2.1±0.3	0.2-0.4	3.5±0.1	+	ND
E-2*	1	M	324	6.1	3.3	2.8**	+	++
E-3*	1	M	325	1.9	1.8	2.2	+	−
E-4	1	M	325	1.7	0.3	5.1	+	ND
F-1, 2, 3, 4, 5	5	F	-	-	-	-	++	ND
F-6*	1	F	-	-	-	-	++	+
Cont (6.5 m. o.)***	6	F	-	1.1±0.3	0.6-0.8	1.3±0.6	+++	−
Cont (7.0 m. o.)	8	M	-	2.3±1.2	0.6	2.0±0.7	+	−

*Rats with weight loss, **Heinz body %: 4.6, ***Previously pregnant rats.

rat IgGAM (H+L) (BSL, England) was mounted on the smears for 45 min. After washing in PBS, the smears were stained using the substrate, naphthol AS-Mx and Fast Red TR. Mayer's hematoxylin stain was followed for 30 sec. One hundred hepatocytes were counted to calculate the percentages of antinuclear antibody positive cells. Auto-hemagglutination: Twenty μl packed RBCs obtained from a syngeneic Lewis male (7 m. o.) were incubated with 30 μl saline containing 3% polystyrene latex beads (Sigma, USA) overnight at room temperature. The latex bead-RBC suspension was diluted to 0.4% RBC with saline. One μl of the 0.4% RBC suspension and 10 μl serum obtained at the time of sacrifice were added to each microplate well (Robbins Scientific, USA). Hemagglutination was examined under the microscope after incubation at room temperature for 50-80 min. The well showed diffusely agglutinated RBC was judged to be positive.

RESULTS

Exp. Nos.-Rat Nos. A-3, B-3, C-1 and D-1 rats were excluded from this study, because these rats died on 236, 233, 300 and 318 days after ferritin injection, respectively. Table 1 summarizes the results of iron metabolisms that were studied in regard to RBC destruction and proliferation. Male rats of A-2, E-2 and E-3 showed respective 49 g, 93 g and 50 g weight losses. Female rats of D-5 and F-6 showed respective 19 g and 21 g weight losses. Reticulocyte count increased to 3.5-6.1% in C, D-5 and E-2 rats. Basophilic erythrocytes increased to 3.3% in the E-2 rat, and siderocytes increased to 9.7% in the A-2 rat and to 5.1% in C-3 and E-4 rats. All of the E rats showed slightly increased numbers of siderocytes (2.2-5.1%). The Heinz bodies of E-2 rat increased to 4.6%. In A-1, 4 & 5 rats, the Heinz body rate was 1.7±0.8%. C-3 rat had 1.2% Heinz bodies on examination at 250 days, and the D-5 rat had 1.5% Heinz bodies. The spleen of the E-2 rat demonstrated extramedullary erythropoiesis. In the rat, iron deposition in the hepatocytes was also enhanced, but the amounts of phagocytized or stored irons were small in the Kupffer cells. The D-5 rat showed enhanced iron deposits in both urinary proximal convoluted tubules and hepatocytes. As shown in Fig. 1, mitochondria were massively burdened with hemosiderin in the proximal convoluted tubules of the D-5 rat. Secondary large lysosomes phagocytized erythrocytes actively in the tubules. Enhanced iron deposition was recognized in the liver of the F-6 rat. RBC uptake in renal tubules was more active in all the experimental and control females than the males. Histopathological changes in the adrenal gland were examined in A-2,

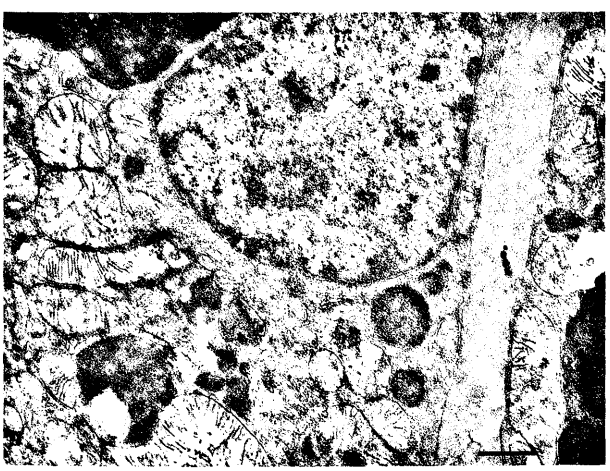

Fig. 1. Electron micrograph of proximal convoluted tubules of a D-5 female, in which massive iron deposits are recognized by light microscopic analysis. Mitochondria are burdened with hemosiderin. One of the secondary lysosomes contains a phagocytized erythrocyte. Two lysosomes as large as nucleus are also seen in the figure. Basal laminas are thicker than normal. Black scale marker indicates 1 μm in length. (Uranyl acetate—lead citrate double stain, ×10,000)

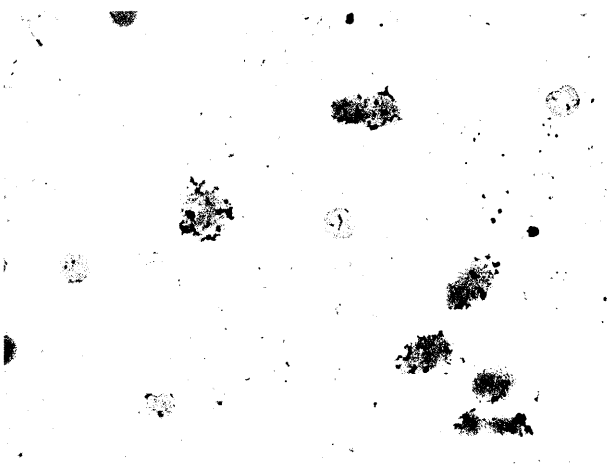

Fig. 2. Hepatic cell smear of an A-2 male. An antinuclear antibody is positive in all nuclei. The IgG antibodies binding with hepatic nuclei are stained red by alkaline phosphatase-conjugated anti-IgG antibody. (Immuno-alkaline phosphatase stain counterstained with Mayer's hematoxylin, ×800)

Table 2. Immunoglobulin assays to hepatic cell nucleus and RBC.

Exp. No.-Rat No.	Sex	Anti-nuclear IgG Ab (Positive hepatocyte %)	Auto-RBC Ab (Hemagglutination)
A-1, 5, 6	M	9, 17, 12	Negative
A-2*	M	92	Weakly positive
B-2, 3, 4	F	11, 86, 21	Negative
C-2, 3, 4	M	10, 59, 21	Negative
D-3, 4, 5*	F	59, 7, 21	Negative
E-1, 3*, 4, 5	M	5, 37, 66, 14	Negative
E-2*	M	9	Positive
F-1, 6*	F	10, 52	Negative
Cont-1, 2, 3, 4	M	5, 6, 8, 2	Negative
Cont-1, 2, 3**	F	2, 6, 19	Negative

*Rat with weight loss, **Previously pregnant rats.

B-2, 3 & 4, C-2, 3, 4 & 5, D-2, 3, 4 & 5, E-1, 3, 4 & 5 and F-1, 2, 3, 4, 5 & 6 rats. Among the female rats, B-4, D-3 and F-5 rats had an atrophic medulla with relatively hyperplastic cortex. The same atrophic medulla was also found in 6 of the 11 control females that had previously given birth to male rats. All the male rats and 8 control males had normal adrenal medullae.

Table 2 summarizes the results of serum immunoglobulin assays. High titers of IgG anti-nuclear antibody to hepatic nucleus were detected in some of the rats. Fig. 2 demonstrates IgG anti-nuclear antibodies to hepatic nuclei, which were stained positively by an alkaline phosphatase-conjugated anti-IgGAM (H+L) antibody. The positive intensity of the anti-nuclear antibody paralleled the numbers of positive nucleus. Ninety-two percentage hepatic nuclei were stained positively in the reaction with A-2 rat serum. Eighty-six percentage hepatic nuclei were positive in the B-3 rat. The E-4 rat showed 66% positive nuclei, while C-3 and D-3 rats had 59% positive nuclei. In the A-2 rat, the auto-RBC antibody was weakly positive. Auto-RBC antibody was identified in the E-2 rat. Auto-antibody to RBC could not be detected in the D-5 rat. Auto-hemagglutination was also negative in the rats of A-4, C-1 & 5, D-1 & 2, F-2, 3 & 4, 7 control females and 4 control males that are not listed in Table 2.

DISCUSSION

Examination of the relations between iron metabolism and autoimmune hemolysis in Lewis rats indicated that a male rat, injected with Wister rat liver ferritin showed an increased number of peripheral siderocytes. Since iron granules are not usually recognized in mature RBCs, one or two small iron granules in 9.7% RBCs means a disturbed heme synthesis in RBC mitochondria. Although autohemolysis was not evident this rat also showed body weight loss with weakness, a high titer of anti-nuclear antibody to hepatic nucleus (Fig. 2) and weak auto-RBC antibody. One female injected with the ferritin was found to have a high anti-nuclear antibody to hepatic nucleus without other obvious abnormalities. The other male that was injected with both ferritin and anti-Ia mAb showed auto-hemolysis. Massive weight loss and auto-RBC antibody were observed in this rat. As 4.6% Heinz bodies were counted in the rat, it was suggested that disturbed heme synthesis also affected the stability of hemoglobin. Siderocyte percentage increased mildly in the 5 males treated with both ferritin and anti-Ia mAb. Disturbed utilization of ferritin in immature RBCs was related to autoimmunity, especially autoimmune hemolysis. T cell receptors (TCR) on $CD4^+$ T helper (or inducer) cells were the most important sites in self-recognition. TCR on helper T cells acts in association with MHC class II molecules on antigen-presenting cells (APC) or macrophages.[6] As immunological disturbances due to iron-overload had been suggested before,[4] it was concluded that self-recognition was disturbed not only by decreased numbers of helper T cells but also by modified MHC class II molecules APC or macrophages.

The other female rat that was injected with ferritin and anti-IA mAb showed enhanced non-transferrin bound iron on liver cells. RBC destruction of the proximal convoluted tubules increased markedly in

this female rat (Fig. 1). Mild hemolysis was transiently suspected in males at 250 days after injection with both ferritin and anti-IA mAb, but non-transferrin bound iron on liver cells was not prominent in the males surviving beyond that time. As it had been stated that antibody to MHC class I antigens block the generation of cytotoxic T cells,[5] autoimmunity was not clear in those rats injected with anti-IA mAb. Experimental autoimmune encephalomyelitis (EAE) was shown to be improved dramatically after the injection of TCR Vβ8, CD2 (39-59) peptide.[6] TCR Vβ8 down-regulated the induction of Vβ8$^+$ T cells, which were specific for the immunodominant 72-89 epitope of myelin basic protein, in association with MHC class I molecules on the Vβ8$^+$ T cells. TCR has reacted to improve EAE in association with MHC class I antigens.

In all the females, RBC destruction in the proximal convoluted tubules was more active than in males. Stimulated production of renal erythropoietin might have regulated the hamopoiesis of bone marrow in females. Adrenal medulla was atrophic in 3 of the iron-overloaded females and 6 formerly pregnant females, but not in male rats. It was shown that phosphocholine-keyhole limpet hemocyanin (PC-KLH) antigen activated the hypothalamic-pituitary-adrenal (HPA) axis.[7] The HPA axis played an important role in B and T lymphocyte immunity to foreign antigens. Synthetic corticosteroid dexamethasone has been reported to regulate the autoimmune disease of the R 16-immunized Lewis rat.[8] Since this study also indicated that autoimmune disturbances were weaker in all iron-overloaded females than males, it was concluded that the activated PHA-renal tubule axis controlled the immunity of self-recognition.

Acknowledgments. The author wishs to thank Mr. Shogo Muranaka for technical assistance with electron micrographs.

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