Suppression of Hemopoiesis during CCl_4-Induced Hepatic Fibrosis: Role of Systemic Endotoxemia

Ya. Sh. Shvarts, A. A. Zubakhin, and M. I. Dushkin

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 130, No. 8, pp. 172-175, August, 2000
Original article submitted May 25, 2000

CCl_4-induced hepatic fibrosis in mice was accompanied by insufficiency of bone marrow myelo- and erythropoiesis. Polymyxin B completely abolished these changes. This phenomenon can be explained by the development of macrophage endotoxin tolerance during hepatic fibrosis.

Key Words: *CCl_4-induced hepatic fibrosis; hemopoiesis; endotoxemia; macrophage; tolerance*

CCl_4-induced hepatic fibrosis (IHF) in mice is accompanied by insufficiency of bone marrow (BM) hemopoiesis manifested by a considerable decrease in the number of granulocyte-macrophage and erythroid precursors, impaired maturation of hemopoietic cells, and tolerance to hemopoietic stimuli [2].

Systemic endotoxemia accompanies diffuse liver diseases and causes various extrahepatic complications including dysfunction of the hemopoietic system [10]. Diffuse postinflammatory hepatic fibrosis leads to suppression of hepatic mononuclear phagocytes [12] and disturbances in blood circulation in the liver and, therefore, is accompanied by persistent endotoxemia. These data suggest that insufficiency of BM hemopoiesis in animals with CCl_4-IHF is related to chronic systemic endotoxemia. To test this hypothesis, we studied the effects of polymyxin B that specifically binds and eliminates endotoxin from the circulation on hemopoiesis in animals with severe CCl_4-IHF.

MATERIALS AND METHODS

Experiments were performed on 95 male BALB/c mice weighing 20-25 g. The animals were obtained from the Stolbovaya nursery and kept in a vivarium under standard conditions and *ad libitum* food and water supply. The mice were divided into 5 groups. Group 1 included intact animals. Group 2 mice received intraperitoneal injections of 5.0 ml/kg sterile olive oil 2 times a week for 16 weeks (control). Group 3 mice were injected with 20% CCl_4 in oil. Group 4 and 5 mice with IHF were daily intraperitoneally injected with 0.05 mg gentamicin and polymyxin B for 7 days, respectively, 3 days after the last injection of CCl_4. Experimental mice were euthanized 10 days after the last injection of CCl_4. We performed 3 independent experiments. Each group consisted of 5-6 animals. All measurements were performed in duplicates or triplicates.

Liver specimens were embedded in paraffin. Fibrosis transformation was verified on histological slices under a microscope.

The total cellularity of the blood and BM was estimated in a Specol cell counter. The number of cells in the blood and BM was differentially determined under an Orthoplan light microscope (Zeiss) using preparations stained by the Pappenheim's method. Blood reticulocytes were counted using preparations stained by brilliant cresyl blue.

The number of granulocyte-macrophage (CFU-GM) and erythroid colony-forming units (CFU-E) was estimated by a modified method described elsewhere [1]. BM cells (10^5) were incubated in 40-mm plastic Petri dishes (Linbro) in RPMI-1640 medium containing 20% fetal bovine serum (Sigma), 5×10^{-5} M 2-mercaptoethanol (Merck), 280 mg/liter glutamine, 50 mg/liter gentamicin, and 0.8% methyl cellulose (Difco). The growth of CFU-E was stimulated with blood serum obtained

Institute of Therapy, Siberian Division of the Russian Academy of Medical Sciences, Novosibirsk

0007-4888/00/0008-759$25.00

from anemic BALB/c mice [4]. The growth of CFU-GM was stimulated by blood serum of BALB/c mice intravenously injected with 100 mg/kg zymosan 2 days before the experiment [2]. The cells were incubated at 37°C and 100% humidity (5% CO_2 and 95% air). CFU-E and CFU-GM were counted on days 3 and 7 of incubation, respectively.

The number of hemopoietic islets was estimated by the method described elsewhere [1]. BM was washed from the femur with 0.5 ml RPMI-1640 medium containing 0.05% collagenase (Merck) and incubated in the same medium at 37°C and 100% humidity (5% CO_2 and 95% air). After incubation, BM was gently resuspended and mixed with an equivalent volume of 0.3% neutral red (Sigma). The number of hemopoietic islets was estimated in a Goryaev chamber. Cell aggregates containing neutral red-stained central cell were referred to macrophage-positive hemopoietic islets. Cell aggregates with unstained central cell were assigned to macrophage-negative hemopoietic islets.

The results were analyzed by Student's t test. The differences were significant at $p<0.05$.

RESULTS

In mice injected with olive oil, we observed only insignificant changes in the blood and BM compared to intact animals (Table 1). The total number of peripheral blood leukocytes and the content of immature BM granulocytes tended to increase. The number of macrophage-positive hemopoietic islets in BM increased by 50%. The content of erythro- and myelopoietic precursors remained practically unchanged. Hence, olive oil stimulated functional activity of macrophages and, therefore, activated erythro- and myelopoiesis, but these changes were statistically insignificant.

In the blood of group 3 mice, we revealed moderate leukocytosis, decreased content of erythrocytes, and more than 4-fold reduction of the number of reticulocytes ($p<0.001$). The total cellularity of BM in mice with CCl_4-IHF did not differ from that in intact and control animals. However, the number of immature neutrophils and monocytes/macrophages in group 3 mice increased by 1.5 and 3.0 times, respectively, compared to that in intact and control animals ($p<0.001$). The content of erythroid elements in these mice decreased by 1.8 times compared to the control ($p<0.001$). Although the total cellularity of BM in mice with CCl_4-IHF did not differ from the control, the content of macrophage-positive elements decreased by 1.5 times (statistically insignificant). The number of CFU-E was 2 times below the control, and the content of CFU-GM tended to decrease. Thus, BM hemopoiesis was markedly suppressed in mice with CCl_4-IHF.

TABLE 1. Parameters of Peripheral Blood and Bone Marrow Hemopoiesis during CCl_4-IHF and Its Correction ($M\pm m$)

Parameter	Intact animals	Oil	IHF		
			without treatment	+gentamicin	+polymyxin B
Peripheral blood					
leukocytes, 10^9/liter	6.80±0.43	7.9±0.9	8.20±0.54	7.60±0.63	12.30±0.98***
erythrocytes, 10^{12}/liter	7.60±0.51	8.10±0.43	6.90±0.54	7.10±0.69	6.70±0.32
reticulocytes, 10^{11}/liter	0.90±0.06	0.80±0.06	0.20±0.04*	0.30±0.05	0.60±0.08***
Bone marrow					
Total cellularity, 10^6/femur	22.00±1.32	23.90±2.13	26.10±1.19	23.00±2.37	24.20±1.85
Immature granulocytes	4.2±0.3	5.10±0.35	6.40±0.52**	6.60±0.75	6.10±0.81
Mature granulocytes	9.50±0.45	10.10±0.44	8.80±0.41	7.90±0.83	10.00±0.09***
Monocytes/macrophages	0.60±0.03	0.80±0.09	1.80±0.07*	1.50±0.11	0.90±0.12***
Erythrocytes	4.60±0.15	4.20±0.13	2.60±0.17*	3.10±0.37	4.20±0.36***
Lymphoid cells	3.50±0.85	2.8±0.5	4.90±0.25	4.20±0.42	2.70? 0.17
Hemopoietic islets, 10^3/femur					
total number	20.20±2.49	31.6±2.3	19.60±2.49	22.20±2.17	43.9±5.7
macrophage-positive	6.3±1.5	9.80±1.18	4.00±1.34	4.50±0.71	7.70±2.79
macrophage-negative	13.9±1.5	21.80±1.85	15.60±1.57	17.70±1.82	36.20±7.33
Colonies, 10^5/BM CFU-E	59.0±7.2	50.0±1.8	38.0±2.1	44.40±2.91	67.0±2.9
CFU-GM	129.00±8.91	126.00±9.87	111.00±9.87	117.00±8.13	155.00±16.26

Note. *$p<0.001$, **$p<0.01$, and ***$p<0.05$ compared to intact mice.

Daily intraperitoneal injections of gentamicin for 7 days produced no significant changes in the peripheral blood and BM. Therefore, all signs of myelosuppression, including disturbed maturation of hemopoietic cells and low contents of myelo- and erythropoietic precursors, were retained (Table 1).

In group 5 mice, parameters of the blood and BM were different, and no hemopoietic insufficiency was found. The total number of blood leukocytes in group 5 mice 1.5-fold surpassed that in group 3 animals ($p<0.01$) primarily due to accumulation of neutrophils and reticulocytes (by 2 times, $p<0.001$). The content of leukocytes sharply decreased during CCl_4-IHF returned to normal. Polymyxin B completely normalized a 1.8-fold decreased content of erythroid cells and a 2-fold increased content of monocytes and macrophages. The number of immature granulocytes changed insignificantly, while the high content of lymphoid cells decreased to the control values. Low number of macrophage-positive islets returned to normal, and the content of macrophage-negative islets even surpassed the control. Hence, the total number of hemopoietic islets increased by more than 2 times. Polymyxin B increased the content of erythro- and myelopoietic precursors. The number of CFU-GM and CFU-E in group 5 mice increased by 1.4 ($p<0.05$) and 1.8 times ($p<0.01$), respectively, compared to that in group 3 mice and even surpassed the control.

Thus, the number of BM myeloid elements markedly increases and the content of CFU-GM decreases during CCl_4-IHF, which indicates impaired maturation of myeloid hemopoietic cells. The number of erythroid elements and macrophage-positive hemopoietic islets (structural and functional elements of erythropoiesis) in BM also decreases. These data indicate severe suppression of erythropoiesis in animals with CCl_4-IHF. Polymyxin B completely abolishes insufficiency of BM hemopoiesis, elevates the content of CFU-GM, CFU-E, and macrophage-positive and macrophage-negative hemopoietic islets, restores the number of BM erythroid cells, and increases the content of peripheral blood leukocytes and reticulocytes.

It should be emphasized that the high content of CFU-GM is associated with minimum changes in the number of immature neutrophils and decreased count of monocytes and macrophages. Together with the increase in the total number of leukocytes, these changes indicate that polymyxin B normalizes maturation of myeloid cells.

Polymyxin B is a cyclic peptide antibiotic that forms complex and inactivates endotoxins (lipopolysaccharides of gram-negative bacteria) [13]. Under normal conditions, endotoxin produced by enteric coliforms and absorbed from the intestine enters the liver through the portal circulation and then is neutralized by liver macrophages. In animals with diffuse hepatitides and liver cirrhoses, endotoxin-neutralizing functions are impaired. Therefore, endotoxin partially enters systemic circulation and causes permanent systemic endotoxemia [10]. Chronic endotoxemia leads to associated multiorgan failure, including hemopoietic disorders.

Endotoxin is a very potent stimulator of mononuclear phagocytes inducing production of various mediators and cytokines with antiinflammatory and hemopoiesis-regulating properties (*e.g.*, granulocyte-macrophage colony-stimulating factor, interleukins IL-1β, IL-6, and IL-12, and tumor necrosis factor-α [5]). At the same time, persistent endotoxemia causes early endotoxin tolerance of mononuclear phagocytes characterized by hyporeactivity of macrophages, low production of antiinflammatory transmitters, and enhanced secretion of antiinflammatory cytokines, including IL-10, platelet-derived growth factor-β, *etc.* Our previous experiments showed that the number of Kupffer cells in the liver sharply decreases during CCl_4-IHF [3]. Decrease in the content of Kupffer cells is always associated with endotoxin tolerance [7]. In our experiments, the development of endotoxin tolerance was confirmed by the absence of lipopolysaccharide-induced production of tumor necrosis factor-α in animals with CCl_4-IHF (pathognomonic sign of tolerance). Independent experiments with chronic CCl_4-induced hepatitis and hepatic fibrosis demonstrated low reactivity of macrophages in the liver, lungs, spleen, abdomen, and other organs [12].

Drastically reduced production of colony-stimulating factors by tolerant mononuclear phagocytes probably accounts for insufficiency of BM hemopoiesis during CCl_4-IHF and stimulation of hemopoietic processes after polymyxin B-induced attenuation of endotoxemia [8,15]. Apart from decreased production of colony-stimulating factors, this phenomenon can be also related to low sensitivity of BM cells, because during endotoxin tolerance intracellular transduction of hemopoietic signals is disturbed due to impaired functions of G-proteins [6], nonreceptor tyrosine kinases [9], transcriptional factors [14], *etc.* Increased production of antiinflammatory cytokines can also contribute to these changes.

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