Morphological Manifestations for the Protective Effect of Miliacin in Organs of Immunogenesis after Treatment with Methotrexate

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Morphological changes in lymphoid organs induced by antitumor drug methotrexate were studied in 219 male (CBA×C57Bl/6)F_1 mice. Plant triterpenoid miliacin attenuated the lymphotoxic effect of this drug.

Key Words: *methotrexate; miliacin; triterpenoids; lymphoid organs*

Folic acid antagonist methotrexate (MT) is extensively used in clinical oncology. MT has immunodepressant activity [10], which limits the use of this drug, decreases the length of treatment, and hinders administration of repeated courses. The search for new compounds attenuating the lymphotoxic effect of MT and increasing the effectiveness of immunorehabilitation after drug treatment is an urgent problem. Recent studies showed that triterpenoids produce a strong immunotropic effect and can be referred to the group of immunocorrectors [4,11]. Plant triterpenoid miliacin was obtained from millet oil. This compound causes hypercellularity of lymphoid organs, stimulates immune response, exhibits antioxidant activity, and decreases lymphocyte apoptosis induced by glucocorticoids [5-9].

Here we studied the effect of miliacin on the recovery of lymphoid populations in central (thymus and bone marrow) and peripheral organs of immunogenesis (spleen) in MT-treated animals.

MATERIALS AND METHODS

Experiments were performed on 219 (CBA×C57Bl/6)F_1 mice. The animals were divided into 4 groups. Group 1 consisted of intact mice (n=14). Group 2 mice (n=74) received MT. Group 3 mice (n=71) received 1.6×10^{-7} mol/kg Tween 21 (solvent of miliacin) in physiological saline after MT treatment. Group 4 mice (n=60) received miliacin after MT treatment. MT was injected intraperitoneally in a single dose of 10 mg/kg. Miliacin in a single dose of 2 mg/kg or solvent was injected intraperitoneally for 3 days (1 h after MT treatment and during the next 2 days). The animals were killed by cervical dislocation on days 4, 7, 10, 14, and 21 after MT injection. Lymphoid organs (thymus and spleen) were weighted and fixed in 10% neutral formalin. Histological preparations were prepared routinely and stained with hematoxylin and eosin. The area of cortical and medullar zones in the thymus, area of T-cell and B-cell zones in the spleen, and cell density in these zones were estimated using a morphometric grid [1]. We prepared 1-5 sections from each organ. Ten fields of view (70×70 μ) were examined in each preparation. Myelokaryocytes were counted in the thigh bone. The relative number of cells from various hematopoietic stems was estimated in bone marrow smears stained by the method of Pappenheim. Not less than 500 karyocytes were counted in each smear.

The results were analyzed by methods of variational statistics (Microsoft Excel, Student's t test).

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RESULTS

Hypoplasia of the thymus was observed 4 days after MT injection. These changes were accompanied by a decrease in the weight of the thymus (by 72.6±2.3% compared to the control), area of medullar and cortical zones in the thymus (by 57.7±2.6 and 73.7±2.1%, respectively), and density of cellular infiltrate (by 41.0±2.5 and 36.4±1.5%, respectively; Table 1). These zones contained cells of different size, which formed disoriented bundles. Most significant changes were revealed in the medullar zone and included fragmentation of nuclei in several cells and appearance of cell conglomerates (5-6 cells) with the formation of 3-4 focuses. Cell density in the medullar and cortical zone slightly increased on day 7. However, the signs of degenerative changes remained unchanged in this period. The degree of changes increased on day 7. Cell density in the medullar zone did not differ from the basal level on day 14. The density of cellular infiltrate in the cortical zone returned to normal only on day 21. However, morphological signs of the thymus were not completely normalized in this per-

TABLE 1. Effects of MT and Miliacin on Morphometric Parameters of the Thymus in (CBA×C57Bl/6)F_1 Mice ($M\pm m$)

Group, period after administration		Weight of the organ, mg	Area of the cortical zone, mm	Cell number in the field of view (490 μ² of the cortical zone)	Area of the medullar zone, mm²	Cell number in the field of view (490 μ² of the medullar zone)
Intact		55.0±2.2 (14)	11.10±0.20 (7/14)	1315.5±24.6 (5/21)	1.96±0.11 (7/14)	858.5±15.3 (5/20)
Day 4	MT	15.1±1.3* (17)	2.89±0.23* (5/10)	837.2±29.1* (5/20)	0.83±0.05* (5/10)	506.6±26.6* (5/25)
	MT+solvent	13.9±0.8* (15)	2.95±0.24* (6/14)	845.90±17.07* (5/21)	0.85±0.07* (6/11)	547.4±20.3* (5/25)
	MT+miliacin	24.2±0.8*°+ (12)	5.00±0.27*°+ (6/15)	1057.4±29.5*°+ (5/25)	1.00±0.09* (6/11)	720.7±27.2*°+ (5/23)
Day 7	MT	21.9±1.4* (21)	3.19±0.38* (5/17)	890.8±26.0* (5/25)	0.91±0.14* (5/17)	591.1±28.8* (5/25)
	MT+solvent	18.25±2.50* (12)	3.27±0.34* (6/26)	880.6±46.3* (5/25)	0.90±0.09* (6/23)	595.0±15.5* (5/21)
	MT+miliacin	30.4±1.4*°+ (15)	6.60±0.33*°+ (8/20)	1100.1±29.1*°+ (7/35)	1.20±0.10* (8/25)	733.4±36.2*°+ (7/35)
Day 10	MT	30.9±2.0* (15)	5.21±0.26* (5/17)	1089.1±15.9* (5/16)	0.96±0.03* (5/10)	635.8±27.4* (5/25)
	MT+solvent	26.6±2.3* (15)	5.42±0.26* (6/15)	1067.6±31.8* (8/25)	0.99±0.10* (6/16)	676.6±21.7* (8/25)
	MT+miliacin	44.1±2.7*°+ (13)	7.21±0.29*°+ (6/15)	1143.6±24.8* (5/22)	1.28±0.07*° (6/16)	782.0±24.5*°+ (5/25)
Day 14	MT	33.9±1.9* (9)	5.56±0.33* (7/15)	1228.6±32.0* (7/22)	1.09±0.06* (7/17)	818.7±20.8 (7/19)
	MT+solvent	36.6±2.6* (12)	5.7±0.5* (6/16)	1230.8±32.2* (5/25)	1.00±0.07* (6/13)	821.7±12.1 (5/12)
	MT+miliacin	49.8±0.9*°+ (8)	7.8±0.5*°+ (7/8)	1357.2±45.3°+ (8/30)	1.30±0.07* (7/12)	918.0±49.8 (8/25)
Day 21	MT	48.3±2.3* (12)	9.40±0.69* (5/10)	1251.2±23.3 (5/25)	1.42±0.16* (5/10)	831.5±15.1 (5/23)
	MT+solvent	46.1±1.4* (12)	9.16±0.67* (5/15)	1247.8±25.4 (5/25)	1.35±0.16* (5/15)	833.0±21.4 (5/25)
	MT+miliacin	55.9±2.0°+ (12)	10.74±0.60 (5/13)	1315.3±21.9°+ (5/19)	1.70±0.16 (5/13)	864.2±12.4 (5/18)

Note. Here and in Tables 2 and 3: denominator, number of sections; value in brackets and numerator, number of animals. $p<0.05$: *compared to intact animals; °compared to MT; +compared to MT+solvent.

iod. The area of zones and weight of the thymus remained below the normal.

Injection of the solvent (Tween 21) after MT treatment had no effect on cell depletion in the thymus, decrease in the weight of the thymus, and subsequent changes in these parameters.

On the 4th day, the decrease in the area of medullar and cortical zones in the thymus (by 47.9±4.8 and 51.5±2.4%, respectively), cell density in these zones (by 16.1±3.2 and 19.6±2.0%, respectively), and weight of the thymus (by 56.0±1.5%) in mice receiving miliacin after MT injection was less pronounced compared to the animals treated with MT alone or in combination with the solvent. Cell disturbances (nuclear degeneration, appearance of cell bundles, and formation of cell conglomerates) were of a rare occurrence in these mice. On day 7, the area of the cortex in miliacin-treated mice was 2-fold higher compared to the animals treated with MT alone or in combination with the solvent. Similar differences were found in the area of the medulla. On day 7 the area of the medulla in animals receiving MT and miliacin was 33.3% higher than in mice of groups 2 and 3. The cells of these zones in group 4 animals had distinct boundaries and similar size. Degenerative changes in the nucleus were not revealed. Similar results were obtained in the follow-up period. The density of

TABLE 2. Effects of MT and Miliacin on Morphometric Parameters of the Spleen in (CBA×C57Bl/6)F_1 Mice ($M\pm m$)

Group, period after administration		Weight of the organ, mg	Area of B-cell zone, mm²	Cell number in the field of view (490 μ² of B-cell zone)	Area of T-cell zone, μ²	Cell number in the field of view (490 μ² of T-cell zone)
Intact		81.2±2.3 (14)	0.126±0.007 (7/40)	938.4±14.3 (5/25)	2426.8±213.7 (7/28)	1227.4±26.0 (5/25)
Day 4	MT	51.5±3.3* (12)	0.069±0.007* (5/25)	598.4±18.7* (5/25)	534.9±96.5* (5/21)	901.0±21.9* (5/25)
	MT+solvent	60.40±2.35* (12)	0.067±0.007* (5/25)	639.2±18.4* (6/25)	558.2±102.5* (5/20)	969.0±15.5* (6/25)
	MT+miliacin	71.1±3.6*°⁺ (12)	0.116±0.009°⁺ (5/23)	782.0±24.0*°⁺ (6/25)	842.6±97.5*°⁺ (5/21)	1077.8±22.3*°⁺ (6/25)
Day 7	MT	80.0±2.5 (21)	0.104±0.008* (5/23)	914.6±28.7 (5/25)	2156.1±279.1 (5/24)	1217.2±24.9 (5/25)
	MT+solvent	76.8±5.5 (17)	0.103±0.008* (5/25)	890.8±25.5 (6/25)	1949.7±233.2 (5/25)	1207.0±27.3 (6/25)
	MT+miliacin	76.4±3.1 (14)	0.117±0.007 (7/22)	911.2±28.8 (6/25)	2267.2±323.4 (7/23)	1196.8±34.0 (6/25)
Day 10	MT	100.0±3.3* (15)	0.128±0.007 (6/23)	1047.2±33.5* (6/25)	2297.9±252.2 (6/23)	1281.8±37.0 (6/25)
	MT+solvent	96.5±2.0* (15)	0.126±0.008 (6/25)	1040.4±34.1* (5/25)	2273.1±209.4 (6/25)	1264.8±21.6 (5/25)
	MT+miliacin	97.7±3.1* (12)	0.126±0.006 (5/23)	1033.6±16.0* (6/25)	2251.8±372.4 (5/21)	1258.0±23.5 (6/25)
Day 14	MT	84.1±4.8 (9)	0.114±0.006 (5/22)	965.6±25.9 (5/25)	2260.4±283.0 (5/25)	1258.0±28.2 (5/25)
	MT+solvent	85.3±4.3 (12)	0.111±0.005 (5/21)	945.2±22.1 (5/25)	2156.4±257.4 (5/25)	1230.8±32.6 (5/25)
	MT+miliacin	86.4±2.6 (8)	0.114±0.005 (5/22)	952.0±32.2 (5/25)	2332.9±273.6 (5/25)	1241.0±39.3 (5/25)
Day 21	MT	74.9±3.2 (12)	0.120±0.008 (5/24)	945.2±37.2 (5/25)	2313.9±285.4 (5/22)	1224.0±42.2 (5/25)
	MT+solvent	76.9±2.6 (12)	0.119±0.007 (5/23)	938.4±38.2 (5/25)	2327.8±281.9 (5/25)	1213.8±44.0 (5/25)
	MT+miliacin	82.2±2.3 (12)	0.121±0.007 (5/21)	945.2±38.7 (5/25)	2363.5±270.9 (5/19)	1234.2±49.8 (5/25)

TABLE 3. Effects of MT and Miliacin on the Number of Myelokaryocytes and Lymphocytes in the Red Bone Marrow of (CBA×C57Bl/6)F_1 Mice (*M*±*m*)

Group, period after administration		Myelokaryocyte number, $\times 10^6$ per femur	Relative number of lymphocytes, %	Lymphocytes, $\times 10^6$
Intact		16.85±0.76 (8)	22.1±1.5	3.70±0.17
Day 4	MT	10.00±0.97* (6)	23.20±1.61	2.28±0.18*
	MT+solvent	11.70±0.81* (6)	20.80±1.45	2.48±0.30*
	MT+miliacin	15.20±0.84^{o+} (6)	17.30±1.45^{*o}	2.59±0.13*
Day 7	MT	14.60±0.95 (7)	19.10±1.68	2.79±0.30*
	MT+solvent	15.80±0.71 (6)	19.30±3.07	3.10±0.55
	MT+miliacin	19.5±0.9^{*o+} (6)	14.00±1.29^{*o}	2.70±0.13*
Day 10	MT	16.30±0.81 (6)	17.00±1.77*	2.73±0.3*
	MT+solvent	16.30±0.55 (6)	17.70±2.91	2.87±0.48
	MT+miliacin	19.7±0.7^{*o+} (6)	18.30±1.45	3.69±0.55
Day 14	MT	16.9±1.0 (6)	24.70±4.07	4.22±0.84
	MT+solvent	16.80±0.45 (6)	25.5±2.1	4.29±0.37

cellular infiltrate in the medullar and cortical zone returned to normal on day 14. The area of these zones and weight of the thymus did not differ from normal on day 21.

On day 4 after MT injection, a decrease in the weight of the spleen (by 36.6±4.0% compared to intact animals) and area of B-cell and T-cell zones in the spleen (by 45.2±4.4 and 77.9±3.8%, respectively, Table 2) was observed. Cell density in these zones decreased by 36.2±1.8 and 26.6±1.8%, respectively. The density of cellular infiltrate returned to normal on day 7. The area of T-cell and B-cell zones increased in this period. The area of the T-cell zone did not differ from the control. However, the area of the B-cell zone remained below the normal (by 17.3±5.6%). On day 10 after MT injection the area of the B-cell zone exceeded the normal by 11.7± 3.2%. These changes were accompanied by an increase in the weight of the spleen on day 10 after MT injection (by 23.2±4.1% compared to the basal level). On day 14, morphological signs of the spleen in treated animals did not differ from those in intact mice.

Administration of the solvent had little effect on these parameters in mice (as compared to MT-treated animals).

On the 4th day, the decrease in the weight of the spleen (by 12.4±4% compared to intact specimens), size of the B-cell zone in the spleen (no significant changes), and area of the T-cell zone in the spleen (by 63.0±2.7%) in animals receiving miliacin after MT injection was much less pronounced compared to mice of groups 2 and 3. Cell depletion in the B-cell zone of the spleen in triterpenoid-treated mice (by 16.7±3.3%) was less pronounced than in the animals treated with MT alone or in combination with the solvent. Miliacin prevented cell depletion in the T-zone of the spleen (in cell number decreased by 12.2±1.8%). Miliacin did not improve morphological signs of the spleen in MT-treated animals during the follow-up period, which was related to the recovery of these parameters in mice of groups 2 and 3. It should be emphasized that miliacin did not prevent the rise in the density of cellular infiltrate in the B-cell zone of the spleen and increase in the weight of the spleen on day 10.

Injection of MT was followed by depletion of the red bone marrow. These changes manifested in a decrease in the number of myelokaryocytes on day 4 (by 40.5±5.7% compared to intact animals,

Table 3). Myelokaryocyte count increased starting from day 7 and was completely recovered by the 10th day.

The solvent had no effect on cell depletion in the bone marrow and subsequent recovery of this parameter.

The protective effect of miliacin manifested in a less significant decrease in the number of myelokaryocytes (9.8±3.5%, day 4) and more rapid recovery of this parameter (day 7). Hyperplasia of the red bone marrow was observed on days 7, 10, and 14 after miliacin injection. The number of myelokaryocytes in treated mice was higher than in intact animals (by 15.7±3.76, 16.7±3.8, and 17.7± 1.7%, respectively).

No significant decrease in the relative number of lymphocytes was found on days 4 and 7 after administration of MT alone or in combination with the solvent. However, the relative number of lymphocytes significantly decreased in group 4 mice receiving MT and miliacin (by 21.6±6.6 and 36.6± 5.8% on day 4 and 7, respectively, compared to intact animals). The relative number of lymphocytes in all animals decreased on day 10, but returned to normal by the 14th day.

It should be emphasized that no decrease in the relative number of lymphocytes in group 2 and 3 animals was accompanied by cell depletion in the bone marrow. Hence, these specific features were not associated with the increase in proliferation and differentiation of lymphocyte precursors in the bone marrow. Our results suggest that this phenomenon is related to migration of extra-bone marrow lymphocytes from the thymus into the red bone marrow. These changes are required for stimulation of suppressed hematopoiesis. This reaction is a typical response of the lymphoid tissue to physical and chemical stress factors (*e.g.*, cytotoxic drugs) with high myeloinhibitory activity [3]. Miliacin probably prevents these changes by decreasing the degree of cell depletion in the bone marrow and thymus. The decrease in the relative number of lymphocytes in group 4 animals on day 10 probably reflects the count of proper karyocytes, since migrating lymphocytes were not identified in the red bone marrow during this period [2]. The recovery of the relative number of lymphocytes and hyperplasia of the red bone marrow in animals receiving MT and miliacin resulted in a significant increase in the absolute number of lymphocytes on day 14.

We conclude that antitumor drug MT has high lymphotoxic activity, which manifested in cell depletion of the bone marrow and lymphoid organs, reduction of the corresponding zones for lymphocyte distribution, and decrease in the density of cellular infiltrate. This effect was particularly pronounced in the T-cell immunity (decrease in test parameters and shortening of suppression). A plant triterpenoid miliacin attenuates the lymphotoxic effect of MT and contributes to a rapid recovery of morphological signs in central and peripheral organs of immunogenesis. These data expand the knowledge of processes that mediate a protective effect of miliacin in the organism. This compound holds much promise as an immunocorrector.

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