PHARMACOLOGY AND TOXICOLOGY

Melphalan in Ultralow Doses Decreases the Severity of Experimental Colitis in Mice

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We studied the effect of melphalan in ultralow doses on mice with experimental colitis induced by substitution of drinking water for 5% dextran sulfate. Daily treatment with melphalan in a dose of 0.025 mg/kg improved the general state of animals. The influence of melphalan was evaluated by quantitative clinical, pathomorphological, and laboratory parameters. Melphalan had a local and systemic antiinflammatory effect.

Key Words: *alkylating agents; melphalan; experimental colitis*

Alkylating agents (cyclophosphamide, chlorambucil, and melphalan) are used as oncostatics and immunodepressants. The cytostatic effect of these drugs is associated with their ability to induce DNA cross-links and breaks (in high concentrations) [5]. DNA is not a unique target for alkylation. However, other potential targets (RNA, second messengers, and surface receptors) do not modulate the cytostatic effect of drugs in doses capable of causing damage to DNA. Previous studies showed that progressive decrease in the dose of drug is accompanied by a decrease in the number of cell targets for alkylation. Under these conditions the drug modulates cell growth, but does not act as a cytostatic agent [12]. Alkylating agents in a concentration of 30 μg/ml (1 order of magnitude below the cytostatic dose of 300 μg/ml) impair interleukin-2 (IL-2) production by activated lymphocytes, which is followed by inhibition of cell proliferation [1,14]. These drugs in ultralow doses (100 times below the cytostatic dose) increase lymphocyte proliferation due to selective damage to the β-chain of the IL-2 receptor (IL-2R) on the surface of regulatory T-lymphocytes [13]. IL-2R is not the only receptor that can be blocked with alkylating agents. For example, maphosphamide in ultralow concentrations protects fibroblastoid cells from the cytostatic effect of tumor necrosis factor-α (TNF-α). This is related to impairment of signal transduction for type I TNF receptors [12]. Probably, alkylating agents in ultralow doses have a positive effect on the course of diseases that are pathogenetically related to inflammation of the mucosa.

Here we studied the effect of alkylating agent melphalan in ultralow doses on mice with experimental colitis.

MATERIALS AND METHODS

Male BALB/c mice weighing 18-20 g were obtained from the nursery of laboratory animals (Cancer Research Center, Russian Academy of Medical Sciences). The animals fed a standard diet and had free access to water. Experimental colitis was induced by substitution of drinking water for 5% dextran sulfate sodium salt (DSS) with a molecular weight of 36-50 kDa (ICN Biomedicals, Inc.). Melphalan (Alkeran®, The Wellcom Foundation Ltd.) served as the alkylating agent. Melphalan was dissolved in

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200 μl phosphate-buffered saline (PBS) and injected intraperitoneally (daily dose 25 μg/ml) from the start of DSS treatment. Control animals received DSS, melphalan, or PBS in the same periods of time.

The mice were daily weighed. They were visually examined for the posture, state of hair, rectal hemorrhage, diarrhea, and blood in the stool. Each parameter was scored (0-4 points) and the total clinical index was calculated as the sum of score for each parameter (maximum 20 points). Objective signs of colitis in 6 animals from each group were evaluated on day 6. The blood was taken from the retroorbital sinus. Leukocyte count and hematocrit were estimated. The animals were killed by cervical dislocation. We measured the length of the intestine and weight of the spleen.

Samples of the proximal portion of the large intestine (2-3 cm from the ileocecal junction) were fixed in 10% neutral formalin, dehydrated, and embedded into paraffin. Serial sections (7 μ) were placed on coded slides and stained with hematoxylin and eosin. A blind study of preparations was performed by 2 investigators [7]. Three independent parameters were evaluated: severity of inflammation (0-3 points); degree of crypt damage (0, no damage; 1, damage to the basal third of the crypt; 2, damage to the basal two thirds of the crypt; 3, complete damage to the crypt, intact epithelium; 4, loss of crypts and epithelium); and depth of damage to the intestinal wall (0, no damage; 1, damage to the mucosa; 2, damage to the mucosal and submucosal layer; 3, transmural injury). The maximum histological index was 10 points.

The results were expressed as $M \pm SEM$. Intergroup differences were significant at $p<0.05$ (Student's t test).

RESULTS

Drinking water with 5% DSS caused colitis in mice, which manifested in diarrhea and appearance of blood in the stool. Changes in the consistency of feces and decrease in body weight were observed on days 3 and 5, respectively. The clinical index in DSS-treated mice was much higher than in animals of the DSS+melphalan group (Fig. 1). The survival rate of DSS-treated mice decreased on day 6 and corresponded to 40% on day 7 (Table 1). Hence, melphalan treatment decelerated the development of colitis symptoms.

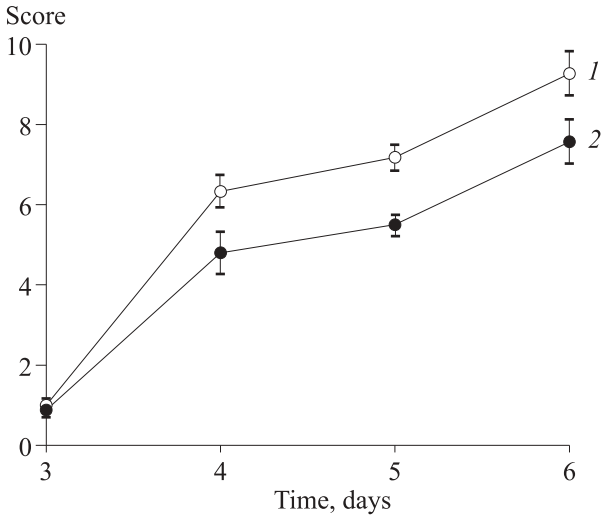

Fig. 1. Clinical index in mice with DSS-induced colitis. DSS-treated mice (*1*); animals of the DSS+melphalan group (*2*).

DSS-treated mice were characterized by a significant decrease in the length of the intestine, severe leukocytosis, increase in the weight of the spleen, and reduction of hematocrit (Table 2). These changes were less pronounced in animals treated with melphalan.

Histological examination showed that the development of colitis is accompanied by serious tissue injury (up to destruction of the wall of the large intestine). We revealed severe edema, massive infiltration with neutrophils and lymphocytes, and

TABLE 1. Survival Rate of Mice with DSS-Induced Colitis ($M \pm SEM$)

Group	Survived animals							
	day 6		day 7		day 8		day 9	
	%	abs.	%	abs.	%	abs.	%	abs.
Control, n=5	100	5	100	5	100	5	100	5
Melphalan, n=5	100	5	100	5	100	5	100	5
DSS, n=15	87	13	40	6	13	2	0	0
DSS+melphalan, n=15	93	14						

Note. n, number of mice.

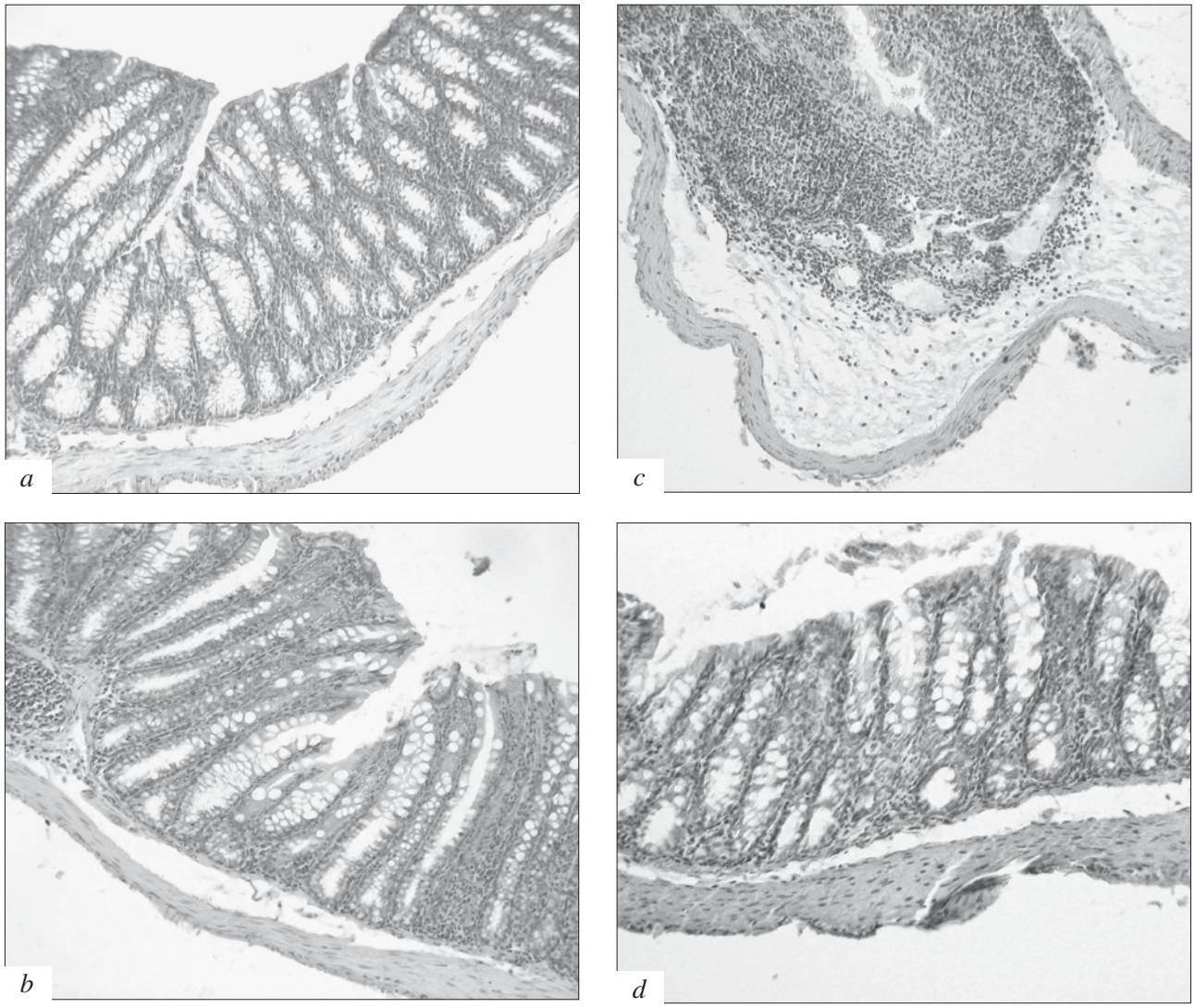

Fig. 2. Pathohistological changes in the large intestine of mice with DSS-induced colitis. Paraffin sections (6 μ), staining with hematoxylin and eosin, ×200. Intact control (*a*); mice receiving injections of melphalan (*b*); mice receiving DSS for 5 days (*c*); mice receiving DSS and melphalan for 5 days (*d*).

loss of the epithelium. The thickness of the mucosa 3-fold surpassed that of the muscle layer. Edema, wall collapse, or complete destruction of crypts were often found. The changes in crypts were more pronounced mice receiving DSS alone compared to animals treated with DSS and melphalan (Figs. 2 and 3).

Histological signs of intestinal damage in animals with DSS-induced colitis were similar to those in patients with Crohn's disease, but differed from pathomorphological characteristics of ulcerative colitis [7]. This experimental model is extensively used in preclinical studies of pharmaceutical preparations [4,7]. We showed that melphalan decelerates the development of DSS-induced colitis in mice. Long-term treatment of the intestinal epithelium with DSS is followed by the impairment of barrier function. Hence, bacteria and their products trigger local and systemic inflammatory reaction. Moreover, phagocytosis of DSS particles stimulates local inflammatory reaction due to stimulation of the *lamina propria* in cells and induction of synthesis of proinflammatory cytokines (IL-1β, IL-12, IL-18, interferon-γ, and TNF-α) [6,8,9,15]. These compounds play a major role in the development of DSS-induced colitis [10,11]. Our previous studies showed that alkylating agents in ultralow doses prevent TNF-α-induced apoptosis in mouse fibroblasts [12]. The effect of melphalan during DSS-induced colitis can be explained by prevention of TNF-α-induced apoptosis in large intestinal epitheliocytes. It should be emphasized that the con-

TABLE 2. Macroscopic Signs of Colitis and Blood Changes in Mice with DSS-Induced Colitis on Day 6 of Study (*M*±*SEM*)

Group	Length of the intestine, cm	Weight of the spleen, % of total body weight	Leukocytes, 10^{-3}/ml	Hematocrit, %
Control	64.3±1.9	0.66±0.03	3.8±1.0	51.2±4.4
Melphalan	64.2±1.1	0.71±0.08	4.1±0.9	49.0±2.1
DSS	51.6±0.8*	0.89±0.08*	13.4±1.3*	48.0±5.5
DSS+melphalan	57.2±1.2*	0.78±0.05	5.9±0.4+	53.0±2.1

Note. $p<0.05$: *compared to the control; +compared to DSS-treated mice.

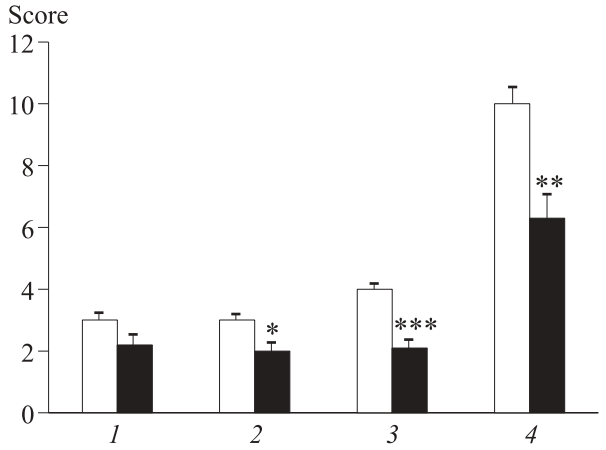

Fig. 3. Histological index in mice with DSS-induced colitis. Severity of inflammation (*1*); degree of crypt damage (*2*); depth of damage to the intestinal wall (*3*); total histological index (*4*). Light bars, DSS-treated mice; dark bars, mice of the DSS+melphalan group. *$p<0.02$, **$p<0.01$, and ***$p<0.001$ compared to DSS-treated mice.

centration of TNF-α progressively increases starting from the 1st day of DSS administration. TNF-α production peaks on days 5-7 [6,15]. Another mechanism of the positive effect of melphalan can be stimulation of regeneration in the epithelium. Previous examination of patients with bronchial asthma inhaling melphalan in ultralow doses provided indirect support for this hypothesis. Histological signs for regeneration of the bronchial epithelium were found in 60% patients [2,3]. The antiinflammatory effect of melphalan in bronchial asthma patients and mice with DSS-induced colitis is probably related to a direct influence of this drug on IL-2R-expressing activated lymphocytes. This assumption is based on the results of *in vitro* studies. We showed that treatment of lymphocytes with alkylating agents in ultralow doses blocks signal transduction in the β-chain of IL-2R [13].

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