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A Combination of Muramylpeptides from Gram-Negative Bacteria Corrects Hematological and Immunological Disorders Induced by Cyclophosphamide

M. V. Kiselevskii[1], N. Yu. Anisimova[1], S. M. Sitdikova[1], F. V. Donenko[1], S. F. Popilyuk[2], V. L. L'vov[3], and O. V. Kalyuzhin[4]

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We have studied the effect of a combination of three natural muramylpeptides containing a *meso*-diaminopimelic acid residue (polyramyl) on the subpopulations of circulating T cells, spleen morphology, and leukocyte level in the blood of C57Bl/6 mice with cyclophosphamide-induced immunosuppression. Intraperitoneal injections of cyclophosphamide in a dose of 100 mg/kg on days 1, 3, 5, and 7 of the experiment reduced leukocyte count and the relative number of $CD4^+$ T cells in the blood, and also depleted the cellular composition of splenic white pulp on day 10. Subcutaneous injections of polyramyl in a dose of 200 μg/mouse on days 8 and 9 practically completely restored blood leukocytes count and morphology of the splenic white pulp. Moreover, administration of polyramyl induced marked tendency to increase in the relative number of $CD4^+$ T cells and CD4/CD8 ratio in mice with cyclophosphamide-induced immunosuppression.

Key Words: *muramylpeptides; polymuramyl; cyclophosphamide; immunosuppression; immunomodulation*

During the past decades, numerous experimental and clinical studies were focused on therapeutic effects of immunomodifiers based on pathogen-associated molecular patterns (PAMP), in particular structural subunits of the bacterial cell wall peptidoglycan (muramylpeptides), in infectious and tumor diseases [6,7]. Several muramylpeptides have already been registered as pharmaceuticals for stimulation of the host immune response against tumors [8] and infections [1]. Some PAMPs possessing pharmacological potential for clinical oncology and hematology are capable of reactivating myelopoiesis and lymphopoiesis suppressed by anticancer chemotherapy. For instance, bacterial multivalent vaccine Immunovac-VP-4 containing various PAMPs (mainly agonists of Toll-like receptors) stimulated hematopoiesis recovery in animals with immunosuppression induced by cyclophosphamide (CP) [2,3]. N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP), agonist of cytosolic NOD2 receptor, dose-dependently increased the level of macrophage progenitor cells in the bone marrow and the level of immature neutrophils and monocytes in the blood [9]. Lipophilic MDP derivative restored leukopoiesis suppressed by antitumor chemotherapy in patients with lung cancer through induction of colony-stimulating factors [5]. A combination of three natural muramylpeptides of Gram-negative bacteria containing *meso*-diaminomelic acid residue and stimulating cytosolic NOD1 and NOD2 receptors (polymuramyl, PM) induced the production of colony-stimulating factors *in vitro* and increased

[1]Laboratory of Cellular Immunity, N. N. Blokhin National Medical Research Center, Ministry of Health of the Russian Federation, Moscow; [2]CORUS Pharma Company, Resident of Biomedical Technologies Cluster, Skolkovo Foundation, Skolkovo, Moscow region; [3]Laboratory No. 16 for Preparatory Biochemistry of Antigens, State Research Center Institute of Immunology, Federal Medial-Biological Agency; [4]Department of Clinical Immunology and Allergology, I. M. Sechenov First Moscow State Medical University (Sechenov University), Ministry of Health of the Russian Federation, Moscow, Russia. ***Address for correspondence:*** kisele@inbox.ru. M. V. Kiselevskii

the level of monocytes and immature neutrophils in the blood of healthy volunteers [4]. At the same time, the effect of this combination on hematopoiesis and differentiation of lymphocytes under conditions of cytostatic-induced immunosuppression has not been studied before.

Here we studied the effect of PM on the subpopulation composition of circulating T cell, spleen morphology, and leukocyte count in the blood of mice with CP-induced immunosuppression.

MATERIALS AND METHODS

Twenty female C57B1/6 mice weighing 22-24 g were purchased from Branch of Stolbovaya Nursery (Research Center of Biomedical Technologies, Federal Medical-Biological Agency). The mice were kept in the vivarium of N. N. Blokhin National Medical Research Center of Oncology, and received standard diet. All manipulations with laboratory animals were conducted in full compliance with the International Guiding Principles for Biomedical Research Involving Animals stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986) and in accordance with the Principles of Good Laboratory Practices (GOST R 53434-2009).

The animals were divided into 4 groups (5 rats per group). For inducing immunosuppression, the mice were injected intraperitoneally with CP (LANS-Farm) in a dose of 100 mg/kg in 0.2 ml sterile water on days 1, 3, 5, and 7 of the experiment. On days 8 and 9, the mice of CP group were injected subcutaneously with 0.5 ml of sterile water (CP group) or PM (Corus Farm) in a dose 200 μg/mouse in 0.5 ml of sterile water (CP+PM group). The animals of PM group received 0.2 ml sterile water intraperitoneally on days 1, 3, 5, and 7 and PM in a dose of 200 μg/mouse subcutaneously. Control animals received sterile water on days 1, 3, 5 and 7 (0.2 ml intraperitoneally) and on days 8 and 9 (0.5 ml subcutaneously).

On day 10, the animals were sacrificed by cervical dislocation in accordance with the accepted guiding principles, the blood was collected individually into test tubes with 3.8% sodium citrate solution (Minimed; 1:1 v/v), the spleens were extracted, and imprints of these organs on the glass slides coated with poly-L-lysine (Menzel-Glazer, Thermo Scientific) were prepared. The smears were fixed in May-Grünwald solution and stained with hematoxylin and eosin (PanEco) by the Romanowsky method.

For evaluation of the phenotype and number of leukocytes, red blood cells were lysed with Opti-Lyse C solution (Beckman Coulter) stabilized with sodium citrate. Leukocytes were counted in a Goryaev's chamber (in 100 large squares). The lymphocyte subpopulations were assessed on BD FACSCanto II flow cytometer (BD Biosciences). Lymphocytes were gated by the ratio of forward (FSC) and side-scatter (SSC) signals after co-incubation of these cells with antibodies to CD4 and CD8 antigens of murine lymphocytes conjugated with fluorescein isothiocyanate and phycoerythrin (eBioscience), respectively. At least 5000 cells were analyzed. The data were analyzed using FACSDiva 6.1.3 software (BD Biosciences). The data were processed using Statistica 10.0 software (StatSoft, Inc.). Significance of differences between qualitative parameters in independent samples was evaluated using nonparametric tests for multiple comparisons were (Kruskal–Wallis test followed by post hoc Dunn's test).

RESULTS

Intraperitoneal injections of CP caused marked leukopenia in mice on the day 10 of the experiment. PM restored the count of circulating leukocytes in CP-treated mice practically to the level of the control group. Subcutaneous administration of PM to intact mice (PM group) did not change significantly the leukocyte count in the blood (Fig. 1).

CP treatment caused cell depletion of the splenic white pulp, which was accompanied by significant reduction of organ size. The borders of the white pulp islands on spleen impression smears were indiscernible, which attested to degradation of the pool of immunocompetent splenocytes. Administration of PM to animals with immunosuppression corrected cellular composition of the spleen: on day 10 of the experiment, multiple assemblies of lymphoid cells composing the white pulp were identified. Injections of the

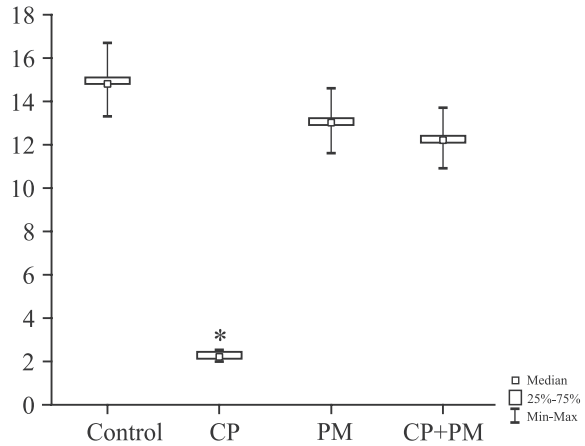

Fig. 1. Effect of PM on leukocytes count in the blood (×10^3 cells/mm^3) of intact and CP-treated mice. p=0.001 multiple comparisons (Kruskal—Wallis test) *p=0.001 in comparison with the control (Dunn's test).

TABLE 1. Effect of PM on the Relative Number and Ratio of T Lymphocyte Subpopulations in the Blood of Mice with CP-Induced Immunosuppression

Parameter	Group	Median	Quartiles		*p* in multiple comparison (Kruskal–Wallis)	*p* in comparison with the control (Dunn's test)	*p* in comparison with the CP group (Dunn's test)
			25%	75%			
%CD4$^+$ cells in the blood	Control	33.8	23.2	37.1	0.016	—	0.217
	CP	7.4	0.3	11.3		0.217	—
	PM	41.4	36.8	49.3		1.000	0.018
	CP+PM	16.4	11.2	23.9		1.000	1.000
%CD8$^+$ cells in the blood	Control	24	21	29	0.181	—	0.254
	CP	48	45	51		0.254	—
	PM	40	37	48		0.992	1.000
	CP+PM	43	41	50		0.489	1.000
CD4/CD8 ratio	Control	1.1	0.95	1.33	0.006	—	0.020
	CP	0.2	0.09	0.21		0.020	—
	PM	1.1	0.87	1.21		1.000	0.068
	CP+PM	0.6	0.33	0.59		0.093	1.000

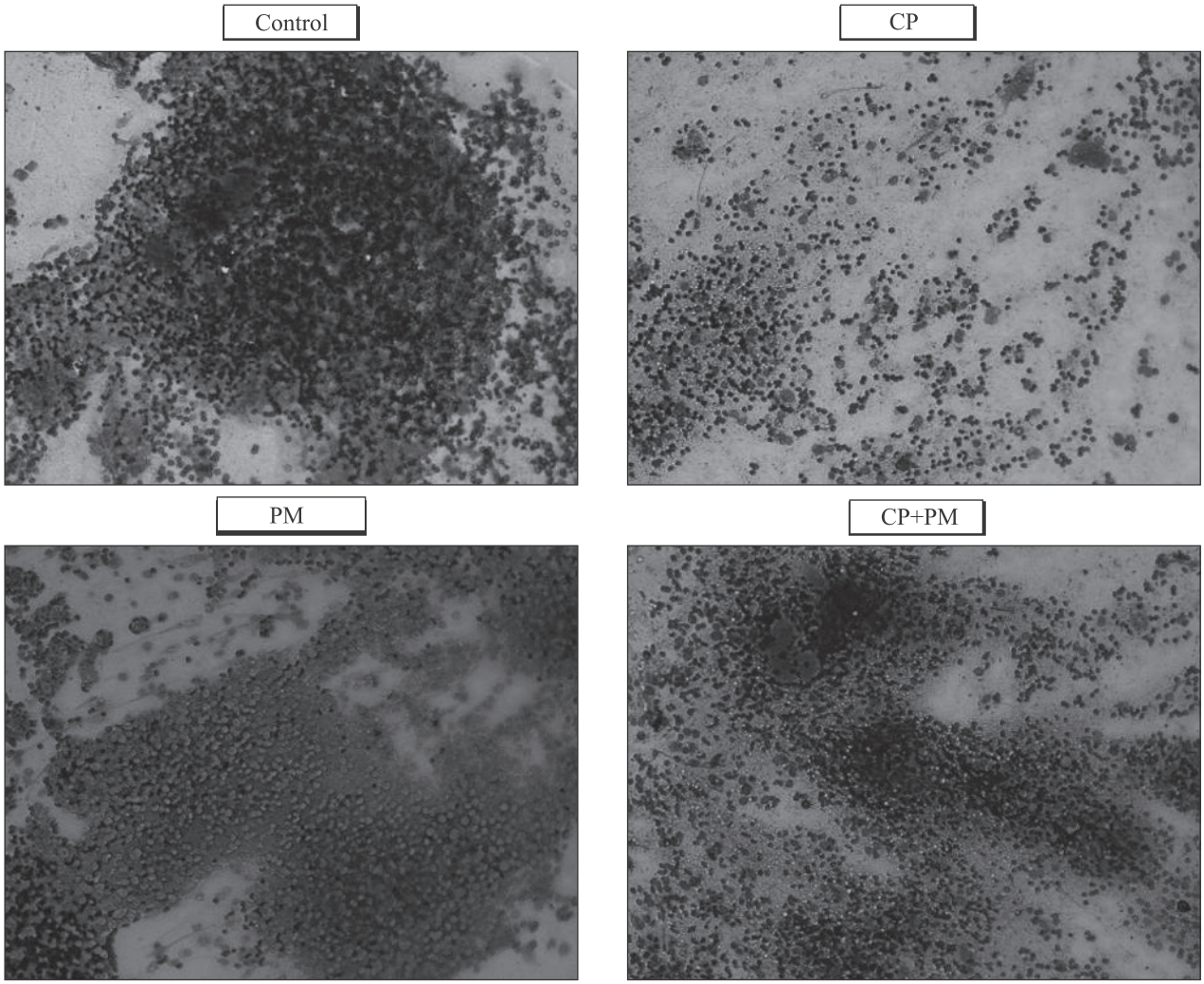

Fig. 2. Spleen impression smears of mice treated with CP and/or PM. Hematoxylin and eosin staining, ×200.

immunomodifier to intact mice (PM group) did not affect the cellular composition of the spleen (Fig. 2).

In CP-treated mice, we observed a 5-fold decrease in the relative number of $CD4^+$ T lymphocytes in comparison with the control group; administration of PM to mice with immunosuppression increased the percentage of these cells by more than 2 times. However the above differences did not reach statistical significance when assessed by Dunn's test, which can be explained by high individual variability of this parameter and small number of animals in the groups (Table 1).

There were no significant shifts of the relative number of $CD8^+$ T lymphocytes, though a certain trend towards an increase in this parameter in CP, PM, and CP+PM groups in comparison with the control group is worthy of note (Table 1).

Multiple comparisons revealed significant intergroup heterogeneity in the CD4/CD8 T cell ratio mainly due to statistically significant 5-fold decrease in this ratio in the CP group in comparison with the control. We observed the trend towards 3-fold increase in CD4/CD8 ratio after PM administration to CP-treated mice. Injections of PM to intact animals did not change CD4/CD8 ratio (Table 1).

Thus, our experiments demonstrated the ability of PM to restore certain parameters of cellular immunity in mice with CP-induced immunosuppression. In particular, PM practically completely restored leukocyte count in the blood and morphology of the spleen white pulp. The relative number of $CD4^+$ T cells in the blood significantly lowered after course treatment with CP tended to increase after 2 PM injections, due to which the CD4/CD8 ratio also tended to increase. The results confirm the advisability of further research of the biological effects of PM in relevant models *in vitro* and *in vivo* for evaluation of the prospects of its use as a hemopoiesis stimulator in cancer patients receiving anti-tumor chemotherapy.

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