ISSN 1063-0740, Russian Journal of Marine Biology, 2015, Vol. 41, No. 5, pp. 389–396. © Pleiades Publishing, Ltd., 2015.
Original Russian Text © O.Yu. Rybalkina, N.N. Ermakova, T.G. Razina, E.P. Zueva, E.G. Skurikhin, M.Yu. Khotimchenko, R.Yu. Khotimchenko, 2015, published in Biologiya Morya.

PHARMACOLOGY AND TOXICOLOGY

Correction of the Toxic Effect of Cyclophosphamide on Hemopoiesis in Animals with Lewis Lung Carcinoma Using Low-Molecular-Weight Sodium Alginate

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Received April 23, 2015

Abstract—The influence of low-molecular-weight sodium alginate, which is administered as an isolated agent and in combination with cyclophosphamide, on the parameters of peripheral blood and bone marrow was studied in mice with Lewis lung carcinoma. It was shown that administration of sodium alginate with a molecular weight of 1–10 and 20–30 kDa to tumor-bearing animals prevents bone marrow failure by activating the process of regeneration of granulocytic hemopoietic stem cells that are damaged by a single injection or repetitive injections of a cytostatic agent, due to stimulation of the clonal activity of granulocytopoiesis precursors. As a result, this treatment prevents the progression of leukopenia.

Keywords: sodium alginate, hemopoiesis, Lewis lung carcinoma, chemotherapy
DOI: 10.1134/S1063074015050119

INTRODUCTION

Marine algae are a source of compounds with unique chemical structures, many of which can be the basis for the invention of new medicinal drugs, biologically active food supplements, and functional products [21, 23, 29]. Alginic acid and its salts (alginates), which are found in marine brown algae and some red algae of the family Corallinaceae, are among the compounds of this kind [24]. Alginic acid and alginates comprise a family of unbranched binary co-polymers that consist of residuals of β-*D*-mannuronic and α-*L*-guluronic acids connected via (1–4)-bonds. Alginates are widely used in the food industry as thickening and gelling agents, emulsifiers, and stabilizers [14]; a large number of works that have been published in the recent decade demonstrate their pharmacological activities. Alginates have been shown to possess radioprotective, hepatoprotective, antioxidant, wound healing, hypocholesterolemic, immunomodulating, and anti-tumor properties [16, 18, 22, 30, 32, 34]. Alginic acid proper, as well as sodium, calcium, and potassium alginates are used both in Russia and in other countries as biologically active food supplements for the correction of gastrointestinal tract disturbances and for removing radionuclides from the body [13].

The use of alginates as agents for the correction of the toxic effects of anti-tumor antibiotics may be a promising trend. In modern clinical oncology, chemotherapy is one of the primary methods of the treatment of malignant neoplasms at each stage of the progression of the tumor process, especially at later stages of extensive metastasis. Administration of cytostatic agents is frequently accompanied by toxicity towards actively proliferating cell populations [15]. Thus, the search for innovative drugs that exert an anti-tumor and anti-metastasis effect, as well as increase the effectiveness of cytostatics and/or decrease their toxicity, is an important task for oncopharmacology. It was shown that plant-derived polysaccharides can increase the effectiveness of chemotherapy and mitigate its side effects [10].

As we demonstrated in preliminary experiments, administration of sodium alginates with low molecular weight to mice with Ehrlich's adenocarcinoma and Lewis lung carcinoma (LLC) results both in inhibition of tumor growth and metastasis and in strengthening of the anti-metastasis effect of cyclophosphamide [8]. The goal of the present work was an experimental study of the effects of low-molecular-weight sodium alginates on the hemotoxicity of cyclophosphamide in order to form a basis for the development of hemocor-

rection agents that could be used in cytostatic therapy of malignant neoplasms.

MATERIALS AND METHODS

The experiment was based on 430 female C57BL/6 mice (2–3 months of age, weighing 19–26 g), category 1, which were provided by the Experimental Biological Model Department of the Goldberg Research Institute of Pharmacology and Regenerative Medicine (RIPRM) in Tomsk (quality certificate no. 188-05). The animals were kept according to the rules prescribed by the European Convention for the Protection of Vertebrate Animals that Are Used for Experimental and Other Scientific Purposes [17]. The experiments complied with Order no. 267 "On approval of laboratory-practice rules," which was issued by the Ministry of Public Health of the Russian Federation on June 19, 2003, and with the "Guide to experimental (pre-clinical) study of new pharmacological substances," Moscow, 2005. The design of the experiments was approved by the Ethics Committee of the RIPRM. At the completion of the experiments the animals were euthanized through dislocation of the cervical spine, according to the "Rules for operations with the use of experimental animals."

Commercial sodium alginate (Sigma, United States) was used as the source material for obtaining experimental specimens of low-molecular-weight polysaccharides. Two specimens of sodium alginate were isolated from it: (A1) with a molecular weight (MW) of 1–10 kDa and (A2) with an MW of 20–30 kDa. In order to obtain oligosaccharides with various MWs, the original sodium alginate was subjected to hydrolysis with 0.5 M hydrochloric acid at a temperature of 90°C for 2, 4, and 12 h with subsequent purification and fractioning of hydrolysis products on ultrafiltration (UF) membranes with the nominal molecular weight limits of 1, 10, 20, and 30 kDa. The A1 specimen was a fraction that passed through 10 kDa UF membranes and was retained on the 1 kDa membrane. The A2 specimen was a fraction that passed through the 30 kDa membrane and was retained on the 20 kDa membrane. A set of pullulans (Sigma-Aldrich, Belgium) and European Pharmacopoeia standards of dextran (Sigma-Aldrich, Strasburg) were used as a calibration standard. The MW of the oligosaccharides was determined by using the method of high-performance liquid chromatography (HPLC) in a Shimadzu LC-20 AD system equipped with a Shodex-Asahipak GS-320 7E column, an RID-10A refractive index detector, and an ELSD-LTII evaporative light scattering detector.

Lewis lung carcinoma (LLC) was inoculated intramuscularly with 5×10^6 cells in 0.1 mL normal saline solution [11]. A1 and A2 were dissolved in distilled water and administered to mice at a dose of 100 mg/kg daily for 12 days, starting from day 7 after inoculation of the tumor, by introducing them into the stomach through a probe tube. Under the conditions of repetitive injections of the cytostatic agent, administration of the studied substances began by the fourth day after inoculation of LLC and lasted 22 days.

Cyclophosphamide, which was used in this work as an alkylating cytostatic agent, possesses a wide spectrum of anti-tumor properties and is included in most combined chemotherapy schemes that have been developed for treating tumors of various geneses [15]. In our experiments, cyclophosphamide (Biokhimik, Russia) was injected to mice once (by the 10th day) intraperitoneally at a dose of 125 mg/kg; in the case of repetitive injections, the cytostatic agent was administered to animals with LLC at a dose of 83.3 mg/kg by the 5th, 12th, and 19th days after inoculation. The animals with the tumor (control) received an equal volume of distilled water, which was introduced into their stomachs through a probe tube, and normal saline solution, which was injected intraperitoneally on the day of administration of the cytostatic agent.

Parameters of peripheral blood and bone marrow were measured in six animals per group by using standard hematological methods [2]. Biological material was collected 1, 3, 5, and 7 days after a single injection of cyclophosphamide (11, 13, 15, and 17 days after tumor inoculation); in case of repetitive injections, by 3, 5, and 7 days after the first, second, and third injection of the cytostatic agent (8, 10, 12, 15, 17, 19, 22, 24, and 26 days after tumor inoculation). The content of granulocyte colony forming units (CFU–G) in bone marrow of mice with tumors was determined through cloning *in vitro* in a semi-viscous culture medium by day 7 after the first injection of the cytostatic agent [2]. The nonparametric Mann-Whitney-Wilcoxon (U) test was used to process the results [6].

RESULTS AND DISCUSSION

At the first stage of the study, the state of the myeloid lineage was studied in mice with LLC that had received sodium alginate with MWs of 1–10 and 20–30 kDa along with a single injection of cyclophosphamide at a dose of 125 mg/kg. As the study showed, leukocytosis progressed in the peripheral blood of mice with tumors starting from the 11th day of the experiment and reached the maximum by the 17th day, predominantly due to the increase of segmented neutrophils (Figs. 1a and 1b). In the bone marrow of mice with LLC, the total number of myelokaryocytes during the experiment was lower than the control level that indicated a depletion of the bone marrow pool (Fig. 1c).

A number of publications have discussed the ambiguous role of neutrophils in oncogenesis: they can exhibit both anti- and pro-tumor activity toward tumors [20, 33]. Stimulation of tumors by neutrophils is manifested most frequently at later stages of growth, when the transformed cells are not identified by the

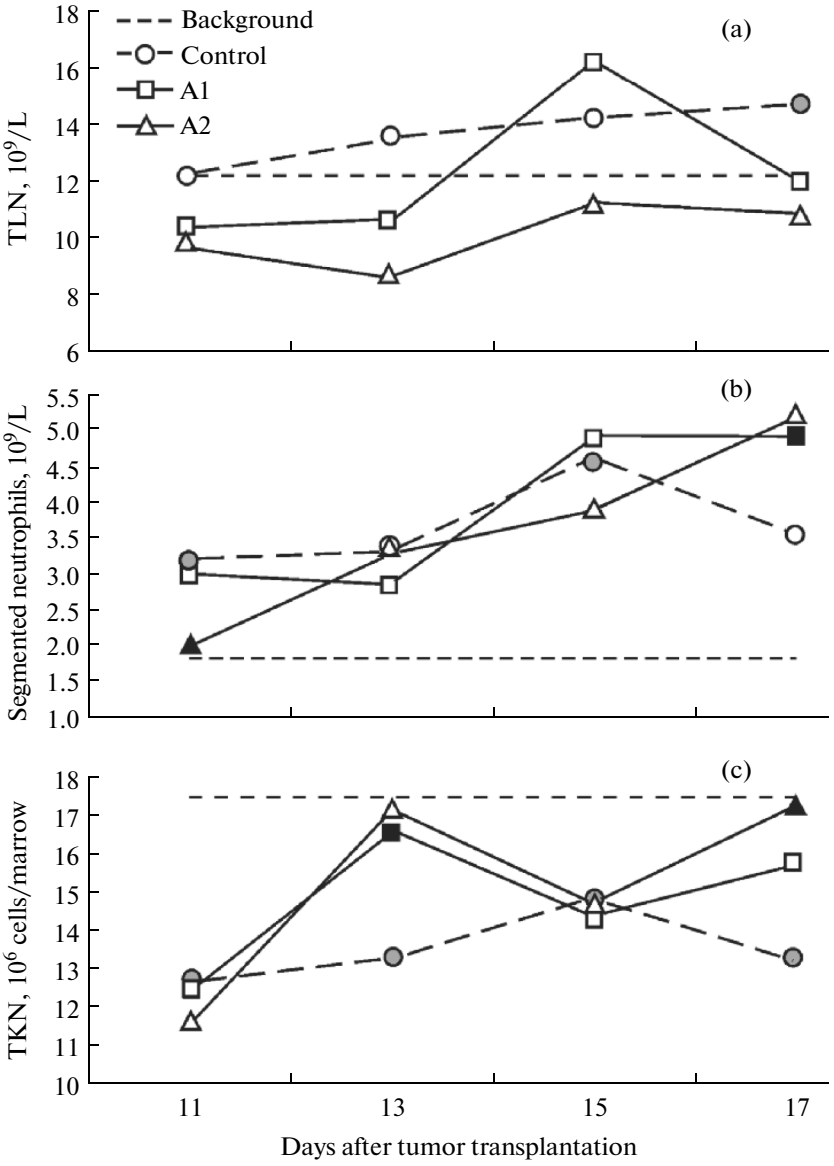

Fig. 1. The dynamics of the total number of leukocytes (TLN) (a), segmented neutrophils (b) in the peripheral blood, and myelokaryocytes (TKN) (c) in the bone marrow of C57BL/6 female mice with LLC via the effects of sodium alginates with molecular weights of 1–10 kDa (A1) and 20–30 kDa (A2). The black symbol is the statistical significance of the difference in the parameter as compared to the control group of mice ($P < 0.05$); the grey symbol is the statistical significance of the difference in the parameter as compared as compared to healthy mice (background) ($P < 0.05$).

immune system [25]. As a rule, this stage of oncogenesis is characterized by extensive metastasis. Okazaki et al. [28] found a relationship between leukocytosis in mice with LLC and stimulation of tumor development. Maltseva et al. [7] showed that the content of neutrophils in the peripheral blood of animals increases at later stages of LLC progression (16–30 days after tumor inoculation), whereas the level of spontaneous and activated production of reactive oxygen species declines, which is evidence of their functional activity; in this case, a deep depression of bone-marrow hemopoiesis is observed [1].

In animals with LLC that received sodium alginate with molecular weights of 1–10 and 20–30 kDa in the isolated form, the degree of manifestation of leukocytosis induced by the development of tumors, decreased (Fig. 1a); the total cellularity of karyocytes in the bone marrow increased by the 13th and 17th days of the experiment, which indicates a decline of the degree of the depletion of the bone-marrow pool recorded from control animals with tumors (Fig. 1c).

In the course of the experiment, pronounced leukopenia was observed in the peripheral blood of mice with LLC by the third day after a single injection of cyclophosphamide at a dose of 125 mg/kg, with a sub-

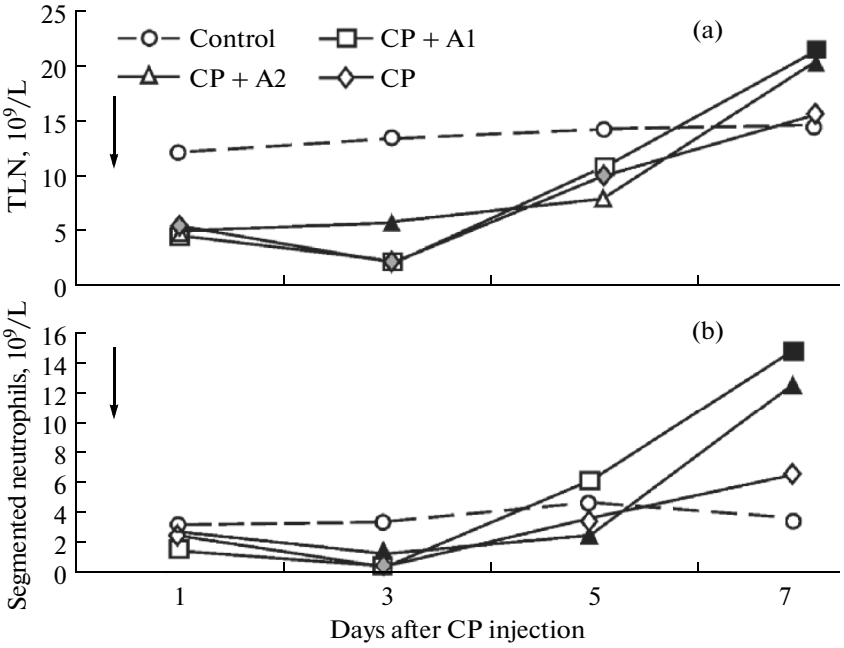

Fig. 2. The dynamics of the total number of leukocytes (TLN) (a) and segmented neutrophils (b) in the peripheral blood of C57BL/6 female mice with LLC via the effects of sodium alginates with molecular weights of 1–10 kDa (CP + A1) and 20–30 kDa (CP + A2) in the case of a single injection of cyclophosphamide (CP). The black symbol indicates the statistical significance of the difference in the parameter as compared to the group of mice that received CP ($P < 0.05$); the grey symbol is the statistical significance of the difference in the parameter as compared to the group of untreated animals (control) ($P < 0.05$). The arrows indicate the days of CP injection.

sequent increase of leukocytes by day 7 (Fig. 2). By 1–5 days after the cyclophosphamide injection, the total number of karyocytes in animals was lower and approached the control level only by day 7 (Fig. 3).

Anti-tumor agents that are used in anticancer therapies do not inhibit tumor growth selectively and exert a side effect on actively renewing cells, thus causing a complex of disturbances [31]. The impact of cytostatic agents on the blood system is considered one of the most hazardous types of toxicity. The pharmaceutical market now offers a number of medicines to decrease the hemotoxicity of cytostatic agents, but their side effects [35], as well as their high price, restrict their wide use. Moreover, as the data that have been collected to date show, some hemostimulating agents facilitate the progression of tumor growth by stimulating angiogenesis [27]. This leads researchers to seek new remedies that can increase the effectiveness of chemotherapy and stimulate hemopoiesis that is inhibited by the cytostatic effect. Marine organisms may be a source of alginates for these medicines.

When sodium alginate with an MW of 1–10 kDa was included in the course of chemotherapy, the total leukocyte number in the peripheral blood of mice with LLC by day 7 after the single cyclophosphamide injection was significantly higher (1.4 times) than the value of this parameter in the animals that had received only the cytostatic agent, due to the larger number of segmented neutrophils (2.3 times) (Fig. 2). With the combined use of sodium alginate with an MW of 20–30 kDa along with the cytostatic agent, the total number of leukocytes in the animals was 2.6 times higher by day 3 and 1.3 times higher by day 7 ($P < 0.05$), mainly due to the increase in the number of segmented neutrophils (Fig. 2).

A myelogram analysis showed that the total number of myelokaryocytes in the bone marrow of mice with LLC that received sodium alginate with an MW of 1–10 kDa, increased by day 7 after injection of the cytostatic agent by 1.4 times ($P < 0.01$) via immature forms of neutrophilic granulocytes and 1.5 times ($P < 0.01$) due to mature ones; this indicates the stimulatory effect of the polysaccharide on granulocytic hemopoietic stem cells. In the case where sodium alginate with an MW of 20–30 kDa was used in the course of chemotherapy, the number of immature and mature neutrophilic granulocytes in bone marrow of mice proved to be higher already by the first day after the cyclophosphamide injection (2.0 and 1.3 times, respectively; $P < 0.05$) (Fig. 3). Thus, including polysaccharides in a chemotherapy course exerts a selective stimulatory effect on granulocytic hemopoietic stem cells that were damaged by a single administration of a cytostatic agent.

Plant-derived polysaccharides exert a broad spectrum of pharmacological effects, including regulation of functions of the immune and endocrine systems, toxin sorption, normalization of lipid metabolism,

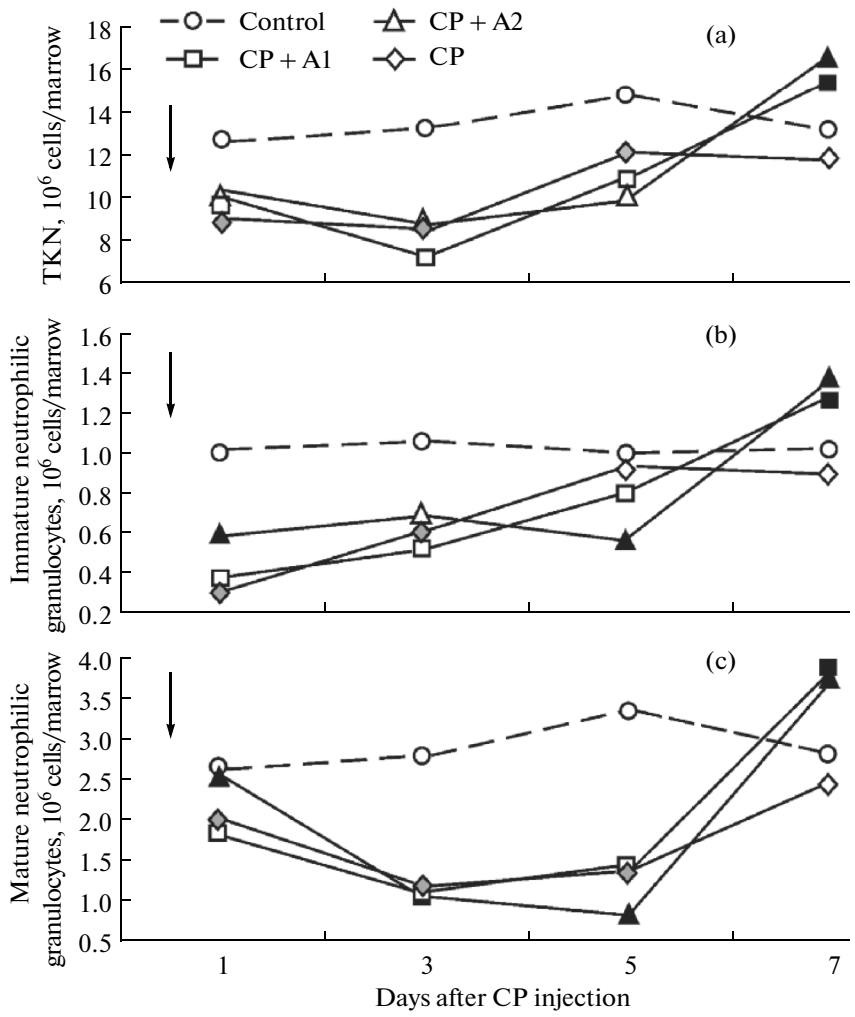

Fig. 3. The dynamics of the total number of myelokaryocytes (TKN) (a), immature (b), and mature (c) neutrophilic granulocytes in the bone marrow of C57BL/6 female mice with LLC via the effects of sodium alginates with a molecular weight of 1–10 kDa (CP + A1) and 20–30 kDa (CP + A2) in the case of a single injection of cyclophosphamide (CP). The black symbol indicates the statistical significance of the difference in the parameter as compared to the group of mice that received CP ($P < 0.05$); the grey symbol is the statistical significance of the difference in the parameter as compared to the group of untreated animals (control) ($P < 0.05$). The arrows indicate the days of CP injection.

and anti-tumor activity [19]. Danilets et al. [4] obtained data on the ability of glycosaminoglycans, which are constituents of water-soluble plant polysaccharides, to alter the functional state of lymphocytes such that they gain anti-tumor properties. The authors believe that polysaccharides inhibit the growth of the primary tumor nodule and metastasis via the activation of M1 cells (classically activated macrophages) and the Th1/Th2 switchover to an effective anti-tumor response (Th1). Medicinal drugs that stimulate the Th1-dependent immunological reactions are used in the immunotherapy of oncological diseases. The mechanism of mitigation of cyclophosphamide hemotoxicity using alginates may be mediated through stimulation of the immune system; it was shown that repetitive injections of sodium alginate to BALB/c mice results in stimulation of the Th1 immune responses and inhibition of the Th2 immune response [5].

For further study, we selected sodium alginate with an MW of 1–10 kDa, which accelerated the restoration of the main parameters of hemopoiesis.

In clinical oncology, chemotherapy is usually conducted in courses, while long-term leukopenia, which appears as a consequence, is an obstacle to the completion of the necessary therapy. Thus, at the following stage of the work, we studied the influence of sodium alginate on hemopoiesis in animals with tumors during a long-term hemodepression caused by repetitive injections of a cytostatic agent. We created a new biological model for moderate inhibition of the growth of the primary tumor nodule and metastasis in mice with LLC accompanied by long-term leukopenia in the

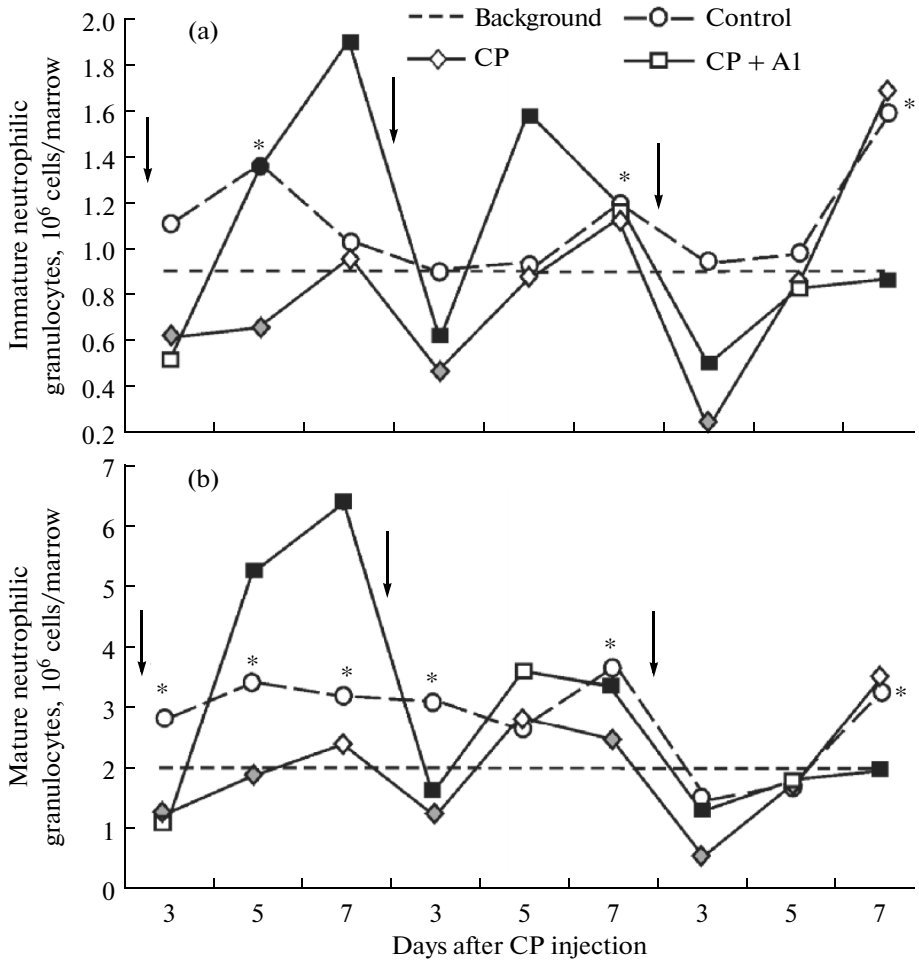

Fig. 4. The dynamics of the contents of immature (a) and mature (b) neutrophilic granulocytes in the bone marrow of C57BL/6 female mice with LLC via the effects of alginate with a molecular weight of 1–10 kDa (CP + A1) in the case of repetitive injection of cyclophosphamide (CP). The black symbol indicates the statistical significance of the difference in the parameter as compared to the group of mice that received CP ($P < 0.05$); the grey symbol is the statistical significance of the difference in the parameter as compared to the group of untreated animals with tumors (control) ($P < 0.05$); asterisk, as compared to healthy mice (background). The arrows indicate the days of CP injection.

peripheral blood with triple cyclophosphamide injections at a dose of 83.3 mg/kg [12].

In experiments with LLC-inoculated mice, the anti-tumor effect of triple cyclophosphamide injections was found to increase due to administration of sodium alginate with an MW of 1–10 kDa [9]. The analysis of hematological parameters showed a decline of the toxic effect of cyclophosphamide on blood cells when it was used in combination with sodium alginate, which was confirmed by a significantly large number of leukocytes and segmented neutrophils in the peripheral blood of mice, both by the fifth day after the first injection of cytostatic agent and by the third day after administration of cyclophosphamide. The analysis of myelograms of animals with LLC showed that in the case of long-term depression of bone-marrow hemopoiesis that was caused by repetitive injection of cyclophosphamide, the use of sodium alginate resulted in growth of the number of mature and immature neutrophilic granulocytes in the bone marrow of mice by day 5 (2.1 and 2.8 times) and by day 7 (2.0 and 2.7 times) after the first injection of the cytostatic agent. A similar situation was observed by the same days after the second cyclophosphamide injection. These results indicate a stimulation of the granulocytic hemopoietic stem. It should be noted that the protective effect of this polysaccharide towards neutrophilic granulocytes was recorded by the third day after the second and third injections of the cytostatic agent to the tumor-inoculated mice (Fig. 4).

An isolated administration of sodium alginate with an MW of 1–10 kDa to animals with LLC resulted in the decline of leukocytosis caused by the progression of tumors. In the bone marrow of mice with LLC that had received sodium alginate in an isolated form, the number of granulocytic neutrophils was noted to increase by the 8th, 10th, 12th, 22nd, and 24th days, while the number of lymphocytes decreased by the

10th, 12th, and 24th days compared to the values of these parameters in the control animals. Thus, under the conditions of repetitive injection of cyclophosphamide, sodium alginate with an MW of 1–10 kDa prevented inhibition of bone-marrow hemopoiesis caused by the cytostatic impact, and facilitated faster recovery of the myeloid hemopoietic stem cells.

The granulocyte colony-stimulating factor, which has an effect on the proliferation and differentiation of progenitor cells of the granulocytic hemopoietic stem cells, plays an important role in the mechanism of hemopoiesis regulation [3]. The objective of the following experiment was to determine the content of CFU-G in mice with LLC when cyclophosphamide was administered in combination with sodium alginate. It was shown that the colony-forming activity of granulocytopoiesis progenitors did not change in animals with tumors that had received only sodium alginate. At the same time, stimulation of CFU-G activity that was inhibited by the anti-neoplastic agent was observed with the combined use of sodium alginate and cyclophosphamide. Thus, the content of granulocytic colony-forming units in a culture of non-adhesive bone marrow cells from mice with LLC increased by 3 times ($P < 0.05$) compared to that in animals that received the cytostatic agent alone. Consequently, one of the mechanisms of the mitigation of cyclophosphamide hemotoxicity via the effects of sodium alginate is its ability to stimulate the fission and maturation of CFU-G in the bone marrow of mice with LLC.

The decisive role in the regulation of the process of proliferation and differentiation of hemopoietic progenitor cells is played by the hemopoiesis microenvironment (HME) [3]. Natural polysaccharides increase the functional activity of HME cells through activation of macrophages and the synthesis of various cytokines that are responsible for the proliferation and differentiation of progenitor cells [26, 36]. In the case of hemodepression caused by repetitive injection of cyclophosphamide at a dose of 83.3 mg/kg, the use of sodium alginate with an MW of 1–10 kDa resulted in stimulation of granulocytic hemopoietic stem cells due to activation of the clonal activity of granulocytopoiesis precursors in the bone marrow of mice with LLC; as a consequence, the number of neutrophilic granulocytes was observed to increase in the bone marrow and peripheral blood.

According to data in the literature, some drugs that are used in clinical oncology for relieving the hemotoxic effects of anti-tumor agents can facilitate tumor growth [27]. Our experiments have demonstrated that low-molecular-weight sodium alginates possess protective and hemostimulatory properties towards granulocytic hemopoietic stem cells that are suppressed by the effect of cyclophosphamide. When administered in isolated form, alginates provide normalization of the content of formed elements in the peripheral blood of mice with LLC, thus preventing bone-marrow depletion. It is important that no stimulation of tumor development was detected due to the use of the alginates; moreover, we observed deceleration of tumor growth, inhibition of the metastatic process, and an increase of the anti-metastatic effect of cyclophosphamide [8, 9].

These results indicate that low-molecular-weight sodium alginates are a promising source for creating pharmaceuticals that mitigate the risk of the development of hemotoxicity in patients with malignant neoplasms who are treated with cytostatic agents.

ACKNOWLEDGMENTS

The work that was related to the isolation and standardization of low-molecular-weight specimens of sodium alginate were conducted at the Far Eastern Federal University and were supported by the Russian Scientific Fund, project no. 14-50-00034.

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Translated by Ye. Shvetsov