In Vitro and In Vivo Effects of Holotoxin A₁ From the Sea Cucumber Apostichopus japonicus During Ionizing Radiation

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Abstract
Radiation therapy is one of the most important approaches to cancer therapy, but radiotoxicity to normal tissue is a serious limitation of this treatment. Compounds which are able to either sensitize cancer cells or protect normal cells to radiation are of great interest. The cytotoxicity of holotoxin A₁ and the effects of radiation against DLD-1 and HT-29 cells were measured by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. The effect of the combination of holotoxin A₁ with X-ray on colony formation of cancer cells was determined by the soft agar assay. The effect of holotoxin A₁ on the recovery of peripheral blood leukocyte number, mass, and cellularity of the lymphoid organs of irradiated mice, as well as on growth of murine Ehrlich solid carcinoma was studied. Holotoxin A₁ enhanced the sensitivity of colorectal carcinoma cells to radiation in vitro. Injection of holotoxin A₁ to mice led to an increase in the spleen endogenous colony number and peripheral blood leukocyte number, as well as the weight and cellularity of the lymphoid organs of the irradiated mice. Holotoxin A₁ in combination with X-ray radiation effectively inhibited the growth of Ehrlich solid carcinoma in vivo. Holotoxin A₁ is suggested to be a promising agent for improving the efficiency of radiotherapy.

Keywords
sea cucumbers, triterpene glycosides, Apostichopus japonicus, acute radiation sickness, spleen endogenous colonies, hematopoiesis

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Radiotherapy is regarded as one of the most important therapeutic treatments of cancer. The standard methods of radiation therapy are limited due to the impossibility of using high doses for the radical treatment of cancer. Under radiation therapy, any attempt to increase the total doses is associated with radiation toxicity to normal tissues and a high risk of severe postradiation damage.¹ That is why finding ways to expand the therapeutic interval of radiosensitivity of normal and tumor tissues or the acceleration and strengthening of the processes of postradiation recovery, primarily in the hematopoietic and immune system, is one of the main strategies to increase the efficiency of radiotherapy. Over the past several decades, radioprotective agents have been developed to protect the host against the side effects of radiation.² For example, amifostine has been used in clinical settings³; however, it causes serious side effects, including nausea.⁴ Recent studies have focused on the development of radioprotective agents derived from natural sources which display minimal side effects on normal cells and tissues.⁵ In this regard, triterpene glycosides of holothurians are of great interest because they possess a wide spectrum of biological activities.⁶ It was reported that cucumarioside A₂-2 from the holothurian Cucumaria japonica possesses immunomodulatory activity by significantly enhancing the natural cellular defense barriers against invading pathogens.⁷ In addition, cucumarioside A₂-2 was shown to induce reversible changes in the macrophage cytoplasm Ca²⁺ concentration, to increase reactive oxygen species formation, the number, size, and acidity of lysosomes of peritoneal macrophages, and to induce some cytokines.⁸

Triterpene glycosides, composed of carbohydrate chains and a triterpene aglycon, are widely distributed in higher plants, many sea cucumbers, and some sponges. Triterpene
glycosides from sea cucumbers preferably have lanostane-type aglycons with an 18(20)-lactone, i.e., related to the holostane series. Holotoxin $A_1$, a triterpene glycoside from the Far Eastern sea cucumber *Apostichopus japonicus*, is a nonsulfated hexaoside with a holostane aglycone having 9(11) and 25(26)-double bonds and a 16-keto group. The carbohydrate chain is branched at the first monosaccharide residue attached to the 3β-O-position of the aglycone. The “top” branch, consisting of 3-O-methylglucose linked to the C-3 position of a glucose residue, is attached to the C-4 of the first sugar (xylose). The “bottom” branch, consisting of 3-O-methylglucose linked to the C-3 of another xylose residue, is linked to the C-4 of a quinovose residue and attached to the C-2 of the first sugar (xylose). All the sugars belong to the D-series and are in pyranose form. All the glycosidic centers have β-configurations.9

Previously, triterpene glycoside preparations called “Cumaside,” a complex of monosulfated triterpene glycosides from *C. japonica* and cholesterol, and a preparation called “Trepangin,” a glycosidic fraction from *A. japonicus*, were shown to increase body resistance and survival rate of irradiated animals. The radioprotective effect was accompanied by an increase in the hematopoietic function, hematopoietic cellularity (bone marrow of the femur), lymphoid (thymus, spleen) organs, and the number of polypotent hematopoietic stem cells.10,11 However, in those studies, the authors used either

![Figure 1. The structure of holotoxin $A_1$ from *Apostichopus japonicus*.](image)

![Figure 2. The radiosensitizing effects of holotoxin $A_1$ from *Apostichopus japonicus* on colony formation in human colorectal carcinoma DLD-1 (a) and HT-29 (b) cells.](image)
mixtures of triterpene glycosides or substances that require repeated administration to achieve a positive effect.

The aim of the present work was to evaluate the in vitro and in vivo radiosensitizing and in vivo radioprotective activities of the triterpene glycoside, holotoxin A1, isolated from the Far Eastern sea cucumber *A. japonicus*, with a precisely defined structure.

**Results and Discussion**

**In Vitro and In Vivo Radiosensitizing Activity of Holotoxin A1**

The cytotoxicity of holotoxin A1 (Figure 1) against human colorectal carcinoma cells DLD-1 and HT-29 was determined by MTS assay. The half-maximal inhibitory concentration (IC50) of holotoxin A1 was 31 and 34 µM against DLD-1 and HT-29, respectively. The dose of radiation (ID50) that caused the death of 50% of DLD-1 and HT-29 cells was 10.6 and 8.5 Gy, respectively. To detect the ability of holotoxin A1 to sensitize colorectal cancer cells to X-ray irradiation, concentrations of 0.1 and 0.5 µM and a dose of radiation of 2 Gy were chosen for further experiments.

Treatment of the investigated cancer cells by X-ray at a dose of 2 Gy was found to inhibit colony formation of DLD-1 and HT-29 cells by 45% and 35%, respectively (Figure 2). Holotoxin A1 (0.5 µM), by itself, slightly inhibited colony formation of DLD-1 and HT-29 cells by 2% and 20%, respectively, compared with nontreated cells (control) (Figure 2). On the other hand, holotoxin A1 (0.5 µM) strongly increased the inhibitory effect of radiation on colony formation of DLD-1 and HT-29 cancer cells by 28% and 52%, respectively, compared with irradiated cells (Figure 2).

Cells (2.4 × 10⁴) were treated with either holotoxin A1 (0.1 and 0.5 µM) or X-rays (2 Gy), or a combination of holotoxin A1 and X-ray radiation, and were then subjected to soft agar. The number of colonies was scored using a microscope with the aid of the ImageJ software program. All experiments were repeated at least 3 times in each group (*n* = 9, with *n* being the quantity of photos). The magnification of representative photos is 10×. Results are expressed as the mean ± standard deviation of measurements. The asterisk (*) indicates a significant inhibition of tumor growth by FeF (**P** < .001).

**Table 1. The Effect of Holotoxin A1 on the Hematopoietic Parameters of X-ray Irradiated Mice.**

| Indicators                          | Control | Irradiated mice, 7 Gy | Irradiated mice treated by holotoxin A1, mg/kg of body weight |
|-------------------------------------|---------|-----------------------|---------------------------------------------------------------|
|                                     |         | 0.1                   | 0.5               | 1                  |
| ECFUS                               | n.d.    | 13.2 ± 4.8            | 36.5 ± 9.6        | 39.1 ± 5.4         | 33.2 ± 10.4 |
| Spleen weight (mg)                  | 77.1 ± 9.1 | 37.3 ± 6.9* | 33.5 ± 5.1b | 37.8 ± 3.4 | 59.0 ± 17.7b |
| Spleen cellularity (10⁹/L)          | 228.3 ± 2.7 | 58.3 ± 11.7* | 53.2 ± 9.2        | 65.6 ± 10.9        | 99.5 ± 11.4b |
| Thymus weight (mg)                  | 45.4 ± 12.3 | 39 ± 3.9             | 45.5 ± 10.9       | 39.8 ± 6.4         | 40.3 ± 10.0 |
| Thymus cellularity (10⁷/L)          | 196 ± 35.12 | 108 ± 31.4* | 157.3 ± 42.7b | 157.3 ± 35.0b | 85.4 ± 9.8b |
| Peripheral blood cell number (10⁹/L)| 5.7 ± 1.7   | 1.2 ± 0.25*         | 2.3 ± 0.8b        | 2.9 ± 0.3b         | 3.04 ± 0.8b |

ECFUS, endogenous colony forming units of spleen; n.d., not determined.

*P* < .05, in comparison with the control group.

**P** < .05, in comparison with the irradiated group.
deviation (SD). The asterisk (*P < .05, **P < .01) indicates a significant decrease in the number of colonies of cancer cells treated with either holotoxin A1 or X-rays compared with the control group or irradiated mice. Figure 4. The effect of holotoxin A1 from *Apostichopus japonicus* on the recovery of peripheral blood leukocyte number (a), spleen mass (b), spleen cellularity (c), thymus mass (d), and thymus cellularity (e) of irradiated mice. Student’s t-test was used to evaluate the data, with a significance level of *P < .05 compared with either the control group or irradiated mice.
PBS-treated cells, or holotoxin A₁ in combination with X-rays compared with irradiated cells.

To the best of our knowledge, this is the first evidence that holotoxin A₁ enhances the therapeutic efficacy of low doses of radiation against human colorectal carcinoma cells, resulting in significant inhibition of cancer cell colony formation. The molecular mechanisms of the radiosensitizing action of holotoxin A₁ have not been investigated in this study. Previously, it was reported that this compound possessed potent antitumor activity in vitro against human primary leukemia K562 cells by the induction of apoptosis. The molecular mechanism of the pro-apoptotic effect of holotoxin A₁ was associated with upregulation of caspase-8 and caspase-3, but not caspase-9 activity. Moreover, sphingomyelinase (SMase) and neutral SMase were activated by holotoxin A₁ in both K562 cells and human primary leukemia cells.

Next, we checked the idea whether holotoxin A₁ (0.5 mg/kg of body weight [b.w.]) was able to sensitize Ehrlich carcinoma (EC) to X-ray irradiation (2 Gy). As illustrated in Figure 3, inoculation of EC cells in the right shoulder area of healthy normal female mice produced a tumor with an average mass of 0.975 g on the 14th day after tumor inoculation. Holotoxin A₁ slightly inhibited growth of the tumor by 13% compared with the control. Exposure of the experimental animals to X-rays 7 days after EC tumor cell inoculation caused a suppression of tumor growth by 48% compared with mice inoculated with EC cells. The combinatory treatment of holotoxin A₁ with radiation led to a significant decrease in the tumor mass by 71% compared with the control. Holotoxin A₁ was thus demonstrated to enhance the inhibiting effect of X-rays on the solid form of Ehrlich carcinoma by 10% compared with irradiated mice (Figure 3).

Previously holotoxin A₁ was found to effectively inhibit the solid form of murine Ehrlich carcinoma and Sarcoma-37. However, this is the first evidence that holotoxin A₁ possesses radiosensitizing activity against Ehrlich solid tumor model.

**In Vivo Radioprotective Activity of Holotoxin A₁**

The dependence of the severity and outcome of acute radiation sickness is associated with the radiation damage of stem cell populations of critical tissues and body systems. The immediate consequence of radiation damage is the cellular emptying of critical organs. Hematopoietic stem cells and the earliest committed precursors of hematopoeisis are distinguished by their maximum radiosensitivities and die within the first few hours and days after irradiation. The decisive moment for a positive outcome of radiation sickness is the speed of recovery of suppressed blood formation, depending on the depth of primary devastation of these populations and the choice of effective treatment aimed at restoring postradiation repair processes.

Cell forming colonies in the spleen of irradiated mice are considered to be equivalent to hematopoietic stem cells. They have huge proliferative potential and are capable of self-maintenance throughout the life of the body. Due to the common stem cells, the hematopoietic and lymphoid tissues are constantly updated.

In the present work, a few endogenous colony forming units of spleen (ECFUS) were found in the spleens of mice under the sublethal doses of X-ray exposure (irradiated group). It was shown that the number of ECFUS of irradiated mice treated with holotoxin A₁ at doses of 0.1, 0.5, and 1 mg/kg of b.w. increased 3.0, 2.8, and 2.5 times, respectively, compared with the irradiated group (Table 1).

The weight of spleen and thymus of mice exposed to X-rays was significantly lower than that of the nonirradiated mice (control group). Holotoxin A₁ (0.1, 0.5, and 1 mg/kg of b.w.) did not have an effect on the weight of spleen and thymus compared with the irradiated group. On the other hand, the administration of holotoxin A₁ in doses of 0.1, 0.5, and 1 mg/kg of b.w. caused increases in thymic cellularity of 25%, 25%, and 18%, respectively, compared with the irradiated group (Table 1).

The obtained results indicated that holotoxin A₁ promoted an earlier restoration of the proliferative function of the blood-forming organs by a significant increase in the number of spleen colonies and thymic cellularity of mice on the ninth day after X-ray irradiation. Previously, some triterpene glycosides from sea cucumbers were shown to possess an immunostimulatory activity at subtoxic concentrations. Glycosides were able to induce the immune cells resulting in an increase in the number of antibody-producing plaque-forming cells in mouse spleens; an increase in the number, size, and acidity of lysosomes of peritoneal macrophages; and an increase in the phagocytic index. It was found that glycosides moderately induce production of some cytokines, restore the level of some CD-markers of lymphocytes, increase bactericidal activity of leucocytes, and induce a significant increase in mouse resistance to lethal doses of some pathogenic microorganisms and radiation.

**Effect of Holotoxin A₁ on the Number of Leukocytes and the Mass and Cellularity of the Lymphoid Organs of Irradiated Mice**

Taking into account the obtained results, we investigated the effect of holotoxin A₁ at a dose of 0.5 mg/kg of b.w. on the dynamics of restoration of the total number of peripheral blood leukocytes, the mass, and the cellularity of the spleen and thymus of mice exposed to X-ray radiation at a dose of 7 Gy.

One day after irradiation, the total number of peripheral blood leukocytes of irradiated mice was markedly decreased compared with the control group (Figure 4(a)). The severity of leukopenia in irradiated mice was greater (up to 10%) than in animals subcutaneously injected with holotoxin A₁ (0.5 mg/kg of b.w.) (up to 22%). By day 15, the number of leukocytes was restored by 29% and 56% in the irradiated group and in the
group of animals that received holotoxin A1, respectively, compared with the control group. Twenty-three to 30 days after irradiation, an increase in the total number of peripheral blood leukocytes of irradiated mice receiving holotoxin A1 was observed. By day 30, the number of peripheral blood leukocytes of mice treated with holotoxin A1 achieved the same level as that of the control groups (Figure 4(a)). It should be noticed that the injection of holotoxin A1 (0.5 mg/kg of b.w.) to mice did not seriously influence the total number of peripheral blood leukocytes (Figure 4(a)).

As shown in Figure 3(b), 1 day after irradiation, the spleen weight of irradiated mice and mice treated with holotoxin A1 decreased by 42% and 36%, respectively, compared with the control group. By day 15, the weight of spleen of irradiated mice treated with holotoxin A1 was found to have increased significantly (Figure 4(b)). In the subsequent periods of the study, on the 23rd and 30th days, the weight of spleen of irradiated mice achieved that of control mice, while it was significantly larger in the group of animals that received holotoxin A1 compared with the irradiated group and control mice (Figure 4(b)).

It was demonstrated that 1 day after X-ray exposure, the content of splenocytes was reduced by 22% in the group of irradiated mice and by 28% in the group of irradiated mice treated with holotoxin A1, compared with the control group (Figure 3(c)). By day 15, the number of nucleated spleen cells in the group of irradiated mice and the group of animals receiving holotoxin A1 recovered to the level of the control group. In the subsequent periods of the study, on the 23rd and 30th days, complete recovery and an additional increase in the spleen cellularity of irradiated mice treated with holotoxin A1 was noted (Figure 3(c)).

The mass of the thymus of irradiated mice and mice treated with holotoxin A1 decreased by 26% and 33%, respectively, compared with the control group. From the 5th to 15th day, the mass of the thymus was restored in groups of irradiated mice treated with holotoxin A1. By day 30, the mass of the thymus of irradiated mice and mice treated with holotoxin A1 was found to have recovered to the level of the control group (Figure 4(d)).

On the first day after irradiation, the number of thymocytes of irradiated mice and irradiated mice that received holotoxin A1 was reduced by 22% and 17%, respectively, compared with the control group. From the 5th to 15th day, thymus cellularity was restored in both irradiation groups, reaching maximum values by the 15th day, while remaining significantly lower than the thymus cellularity in control mice. From the 23rd to 30th day, the number of thymocytes decreased in groups of irradiated mice and irradiated mice treated with holotoxin A1 (Figure 3(e)). We may assume that under certain circumstances (X-ray exposure), the thymus undergoes acute thymic involution (alternatively called transient involution), which is characterized by the shrinking of the thymus, resulting in changes in the architecture of the thymus and a decrease in tissue mass.20

Triterpene glycosides are known to be low molecular weight bioregulators that effectively regulate the ionic permeability of biological membranes of various types of cells and modulate calcium transport.21 As a result of this effect, a variety of cellular functions are enhanced and stimulated, including activation of cell division and stimulation of immunocompetent cells, which, apparently, can underlie the manifestation of biological effects such as the mitogenic and immunostimulating activity of the studied compounds. An increase in glycoside concentration leads to sharp changes in the permeability of macrophage biomembranes for Ca2+ and subsequent inhibition of cellular functions. It was shown that high concentrations of Ca2+ are poisonous to cells and lead to disruption of the functional activity of the cell, “calcium shock,” and cell death.22 The reduction in the thymus weight and thymus cellularity of mice treated with holotoxin A1 may be a consequence of the toxic effect of the drug on the permeability of cell biomembranes.

Conclusion

Holotoxin A1 at subtoxic concentrations was found to significantly sensitize colorectal carcinoma cells to X-ray radiation in vitro. Holotoxin A1 enhanced the inhibiting effect of X-ray radiation in mice with the solid form of Ehrlich carcinoma. Moreover, in vivo administration of holotoxin A1 led to the activation of cell division processes and hematopoietic and lymphoid tissue renewal, resulting in an increase in spleen endogenous colonies and complete restoration of the peripheral blood leukocyte number. Holotoxin A1 induced an increase in mass and cellularity of the spleen, but not the thymus of irradiated mice that can be associated with acute thymic involution. Thus, we may suggest that holotoxin A1, isolated from the sea cucumber A. japonicus, might be promising for radiosensitizing cancer cells and acting as a radioprotective agent; however, the elucidation of the molecular mechanism of its action is necessary.

Experimental

Reagents and Chemicals

Organic solvents, inorganic acids, and salts were commercially available from “Laverne-Lab” (Moscow, Russia). McCoy’s 5A and RPMI-1640 mediums, fetal bovine serum (FBS), L-glutamine, and penicillin-streptomycin were obtained from “Biolo” (St. Petersburg, Russia). The CellTiter 96 nonradioactive cell proliferation assay kit was purchased from “Promega” (Madison, WI, United States).

Triterpene Glycoside

Holotoxin A1 was isolated from the sea cucumber, A. japonicus (Selenka), collected in Peter the Great Bay (Sea of Japan) on May 2018 by SCUBA diving at a depth of about 5 to 10 m using the procedure common for sea cucumber glycosides.23
The sea cucumber specimens were minced and extracted twice with 60% EtOH under reflux. The extracts were evaporated to dryness, redissolved in water, and chromatographed on a Polychrom-1 column (powdered Teflon, Bioral, Latvia). The inorganic salts and impurities were eluted with water, followed by elution of a crude glycosidic fraction with 50% EtOH. Holotoxin A1 was isolated from the glycosidic fraction by Si gel column chromatography using the solvent mixture of CHCl3/EtOH/H2O (100:50:4) as a mobile phase. The establishment of the structure of holotoxin A1 was carried out using 13C NMR spectroscopy by comparing the spectral data with the data published in the literature.

In Vitro Assays

Cell culture. The HT-29 (ATCC # HTB-38TM) and DLD-1 (ATCC # CCL-221TM) human colon cancer cell lines were cultured in McCoy’s 5A and RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS and 1% penicillin-streptomycin in a humidified atmosphere containing 5% CO2.

Cell Cytotoxicity Assay

Cells (3 × 10⁴) were seeded in 96-well plates in 200 µL of the appropriate medium and incubated at 37°C in a 5% CO2 incubator. After 24 hours, the medium was removed and replaced with fresh medium containing different concentrations of holotoxin A1 (from 0.1 to 50 µM) for an additional 24 hours at 37°C in a 5% CO2 incubator. MTS reagent (15 µL) was added to each well, and cells were incubated for 4 hours at 37°C and 5% CO2. Absorbance was measured at 490/630 nm.

X-ray Exposure

To expose the cells to X-ray radiation, an XPERT 80 X-ray system (KUB Technologies, Inc, Milford, CT, United States) was used. A DRK-1 X-ray radiation clinical dosimeter (AxlWant LLK, Moscow, Russia) was applied to measure the absorbed dose of radiation.

Cell Irradiation

To determine the cytotoxic dose of X-rays, cells (5.0 × 10⁵) were seeded in 60 mm dishes. After 24 hours, the cells were exposed to X-rays at a dose rate from 2 to 10 Gy. Then, the cells were recovered at 37°C in a 5% CO2 incubator for 3 hours. The cells were harvested with 0.25% trypsin/0.05 M EDTA solution and subjected to the cell cytotoxicity assay.

To determine the combinatory effect of holotoxin A1 and radiation, cells (5.0 × 10⁵) were seeded in 60 mm dishes and incubated for 24 hours. After incubation, the cells were treated with holotoxin A1 (0.1 and 0.5 µM) for an additional 24 hours. Then, the cells were exposed to X-rays at a dose of 2 Gy. Immediately after irradiation, cells were returned to the incubator for recovery. Three hours later, the cells were harvested and used for the soft agar assay, as described below.

Soft Agar Assay

HT-29 and DLD-1 cells (2.4 × 10⁴/mL), irradiated and treated with holotoxin A1, were grown in 1 mL of 0.3% basal medium Eagle’s agar containing 10% FBS. The culture was maintained at 37°C in a 5% CO2 incubator for 2 weeks. Cell colonies were scored using a microscope and ImageJ software bundled with 64-bit Java 1.8.0_112 (NIH, Bethesda, MD, United States), as previously described.

In Vivo Assay

Animals. Male mice C57BL/6, 6 weeks old and weighing 18 to 20 g, were delivered from the “Nursery for laboratory animals” (Pushchino, Russia) and used for the experiments. The work was performed in compliance with the rules and international recommendations of the European Convention on the Protection of Vertebrate Animals used for experiments or for other scientific purposes (Strasbourg, 1986). All mice were randomly divided into groups with 6 or 10 animals. Before the experiments, mice were quarantined for 2 weeks.

Determination of Acute Toxicity of Holotoxin A1

In order to determine LD50 (the dose of the tested compound required to kill half the number (50%) of test animals), holotoxin A1 was dissolved in PBS and intraperitoneally (i.p.) injected into mice at 0.1, 0.5, 1, 5, 10, and 50 mg/kg of b.w. (10 mice per dosage group).

Ehrlich Tumor Cells Inoculation

Ehrlich ascites carcinoma cell line was maintained in the experimental female C57BL/6 mice by weekly i.p. injection of 2.0 million cells per mouse. The cells were counted before i.p. injection using a hemocytometer, and dilution was made using physiological sterile saline solution. The desired numbers of cells were injected in a volume of 0.2 mL. To assess Ehrlich solid tumor, 0.2 mL cells (2.0 × 10⁶ cells/mouse) were inoculated subcutaneously into the right shoulder area of healthy normal female mice.

Experimental Design

Twenty five animals were randomly divided into 5 equal groups as follows:

- Group 1: Normal control group.
- Group 2: Animals were inoculated subcutaneously in the right shoulder area with Ehrlich carcinoma cells (2.0 × 10⁶ cells/mouse).
- Group 3: EC-bearing mice were treated i.p. with holotoxin A1 (0.5 mg/kg of b.w.) 3 times in 7 days after inoculation with EC.
- Group 4: EC-bearing mice were exposed to whole body X-ray radiation at a dose level 2 Gy once in 7 days after inoculation with EC.
Group 5: Seven days after inoculation of EC, EC-bearing mice were exposed to whole body X-ray radiation at a dose level 2 Gy once and in a day were treated by holotoxin A1 (0.5 mg/kg of b.w.) 3 times in a week.

Mice Irradiation
Holotoxin A1 was i.p. injected into mice at doses of 0.1, 0.5, and 1 mg/kg b.w. (6 mice per group) 24 hours prior to radiation. Mice were placed in a specially designed, well-ventilated acrylic container and subjected to whole-body irradiation by 7 Gy (dose rate of 0.36 Gy/min, voltage of 50 kV, and current of 1 mA) in a single fraction. The absorbed dose was measured using an X-ray radiation clinical dosimeter (DRK-1, Axelbant LLK, Moscow, Russia).

Determination of the Number of Endogenous Spleen Colonies
C57BL/6 mice were intraperitoneally injected with holotoxin A1 and exposed to X-ray irradiation, as described above. On the ninth day after irradiation, animals were sacrificed using anesthesia; spleens were removed, fixed in Carnoy's solution without chloroform (EtOH/glacial acetic acid: 3/1), and then the number of spleen colonies was calculated. In parallel, the number of peripheral blood leukocytes was calculated, the number of spleen colonies was calculated. In parallel, the number of peripheral blood leukocytes was calculated, the mass of the spleen and thymus was measured, and the number of nucleus-containing cells of the thymus was determined in all experimental groups. For this purpose, the spleen and thymus were removed, weighed, homogenized in PBS, and then filtered through a nylon filter, and the number of nucleated cells was counted using a hemocytometer.

Determination of the Effect of Holotoxin A1 on the Hematopoietic Cell Recovery of Irradiated Mice
C57BL/6 mice were treated with holotoxin A1 at a dose of 0.5 mg/kg of b.w., and after 24 hours, they were exposed to X-rays at a dose of 7 Gy. Mice received only an equivalent amount of PBS (control group) or received PBS and X-ray irradiation (7 Gy) (irradiated group). Animals were sacrificed using anesthesia on the 1st, 5th, 15th, 23rd, and 30th days after X-ray irradiation, and the numbers of peripheral blood leukocytes, splenocytes, and thymocytes were counted. The weight and cellularity of lymphoid organs (thymus and spleen) were also measured, as described above.

Statistical Analysis
In vitro assays were performed in at least 3 independent experiments. The results are expressed as the mean ± SD. Student's t-test was used to evaluate the data with the following significance levels: *P < .05, **P < .01, ***P < .001. Data analysis of in vivo assays was performed using the software package “Statistica-10.” For all data, arithmetic averages and their SDs were calculated. To determine the significance of intergroup differences, one-way variance analysis (ANOVA) was used.

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