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Administration of the bacterial extract Broncho-Vaxom® enhances radiation recovery and myelopoietic regeneration

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Abstract

In the present study, we show that the bacterial extract Broncho-Vaxom® (BV, 500 μg/mouse; free of endotoxin) has radiation recovery activity when administered i.p. 24 h before sublethal irradiation. In the postirradiation period (5–12 days), pretreatment of mice with BV induced significantly increased bone marrow cellularity and accelerated myelopoietic regeneration (committed progenitor granulocyte-macrophage colony-forming cells; GM-CFC) in the bone marrow compared with saline-treated controls. The earlier hemopoietic recovery in BV-injected mice was not associated with an increase in the number of bone marrow GM-CFC and CFU-S (colony-forming units-spleen) within 24 h after injection. Simultaneously, a significant diminution in bone marrow cellularity occurred. In addition, the percentage of both GM-CFC and CFU-S in the S-phase of the cell cycle was significantly increased 24 h after a single treatment. In our experiments colony stimulating activity (CSA) in the serum of treated mice was not observed within 24 h after injection. Administration of BV 24 h prior to lethal irradiation, resulted in an increase in the number of surviving mice. Combined administration of BV (24 h) and indomethacin (24 h and 3 h) to mice, prior to irradiation, caused an additional radioprotective effect. These results demonstrate that BV stimulates myelopoietic regeneration and suggest a mechanism by which this treatment protects mice from otherwise lethal irradiation.

Key words: Radioprotection; Broncho-Vaxom®; Indomethacin; Hemopoiesis

1. Introduction

Immunomodulators, either microbial agents or recombinant cytokines, can also enhance survival, hemopoietic and functional cell recovery after irradiation. It has been suggested that these agents mediate radioprotective effects by mechanisms such as enhancing the proportion of hemopoietic stem cells in less radiosensitive phases of the cell cycle, increasing the size of the preirradiation stem cell pools, and accelerating restoration of functional hemopoietic cell populations (Ainsworth, 1988; Chirigos and Patchen, 1988).

Broncho-Vaxom® (BV), lyophilized fractions of bacterial extract (endotoxin-free) from eight strains (Mauel et al., 1989), is used as a polyvalent immunotherapeutic agent in the treatment of respiratory

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tract infections, particularly acute and chronic bronchitis (Maestroni and Losa, 1984; Palma-Carlos et al., 1987; Heintz et al., 1989). The non-specific immunostimulant properties of BV have been demonstrated by several investigators. For example, it is known that it can stimulate the mitogenic response of peripheral blood leukocytes to polyclonal activators (Clot and Andary, 1980) and to allogeneic lymphocytes (Maestroni and Losa, 1984). Furthermore, it has been found to stimulate immunoglobulin production in sputum and serum (Puigdollers et al., 1980), to increase the number of phagocytes, and to enhance the resistance against experimental infection by streptococci and staphylococci in immunosuppressed animals (Bosch et al., 1984). Preincubation of a macrophage cell-line with this extract has been reported to induce interleukin-1 (IL-1) secretion and production of prostaglandins (Bottex et al., 1988).

Our previous studies demonstrated that BV injection before irradiation increased the number of endogenous colony-forming units (E-CFU) in sublethally irradiated mice and increased the number of mice that survived beyond 30 days after lethal irradiation (Fedoročko et al., 1992; Fedoročko and Brezáni, 1992). Also, BV injection accelerated early recovery of cells in the peripheral blood (Macková and Fedoročko, 1993) and CFU-S in the bone marrow. However, the earlier recovery of CFU-S in BV-injected mice was not associated with an increase in the number of CFU-S surviving immediately after irradiation (Fedoročko et al., 1992).

The aim of this study was to characterize the myelopoietic properties of BV and to evaluate the in vivo effects of preirradiation injection of BV on the recovery of hemopoietic damages from sublethal dose of irradiation. We investigated CSA of the serum and lung, the numbers of GM-CFC and the proportion of both bone marrow GM-CFC and CFU-S in the S-phase of the cell cycle of normal mice that occurred within 24 h of a single radioprotective dose of BV. We also studied bone marrow cellularity and recovery of the number of GM-CFC in sublethally irradiated mice. In addition, we presented evidence that joint administration of BV and indomethacin exerted an additional radioprotective effect.

2. Materials and methods

2.1. Mice

Female C57B1/6 mice, 8–10 weeks old, were obtained from Velaz (Prague, Czechia). Animals were quarantined for a period of 2 weeks. They were housed in rodent cages, five to seven animals per cage at about 22°C, and were given Velaz/Altromin 1320 St laboratory chow and tap water acidified to pH 2.4 ad libitum. Research was conducted according to the principles enunciated in the Guide for the Care and Use of Laboratory Animals, prepared by the State Veterinary Office of the Slovak Republic, Bratislava.

2.2. Treatment with Broncho-Vaxom®

BV (Biogal Pharmaceutical Works, Debrecen, Hungary, under licence from OM Laboratoires, Geneva, Switzerland) is a lyophilized extract of the eight most common bacteria of the upper respiratory tract (Fedoročko et al., 1992) and free from endotoxins (less than 0.0002% by *Limulus* and pyrogenicity tests; Bottex et al., 1988). Immediately before use, the drug was resuspended in saline in a volume of 0.4 ml and administered intraperitoneally (i.p.) 24 h before irradiation at a dose of 500 μg per mouse. Control animals received i.p. saline in the same volume and at the same time as the treated group.

2.3. Indomethacin treatment

Indomethacin (Sigma Chemical Co., St. Louis, MO, USA) was prepared by dissolving 10 mg in 1 ml of 95% ethyl alcohol. This solution was then diluted to the working concentration with Dulbecco's phosphate-buffered saline (TechGen Int. Ltd., UK) and injected i.m. at 40 μg per mouse, in a volume of 0.2 ml 24 and 3 h before irradiation. Both of the drugs were given either alone or in combination.

2.4. Irradiation

Mice were placed in plexiglass containers and whole-body (unilaterally) exposed to 6.0 Gy of gamma rays at a dose rate of 0.4 Gy/min, 24 h after

saline or BV injection. A Chisostat ^{60}Co source (Chirana, Czechia) was used for all irradiations.

2.5. Survival

Survival was monitored daily and was reported as percentage of animals surviving 30 days after irradiation. Ten mice per group were used in each experiment. Moribund animals in this experiment were killed. On day 31, surviving mice were killed by cervical dislocation. Experiments were repeated two times. Survival rates were compared among groups using the chi-square test including Yates' correction.

2.6. Hemopoietic stem cell assays

Two primary assays were used to assess the radioprotective effects of BV on hemopoietic stem cells. They include in the in vivo exogenous (CFU-S) spleen colony-forming unit assay (Till and McCulloch, 1961) and the in vitro granulocyte-macrophage (GM-CFC) progenitor cell assay (Vacek et al., 1991).

Determinations of CFU-S were done basically as described by Till and McCulloch (1961). Groups of mice ($n = 5$) were given either 500 μg of BV per mouse or saline alone i.p. 24 h later, both femora were removed and a bone marrow cell suspension was made. Cells were flushed from femurs with 2 ml IMDM containing 15% heat-inactivated newborn calf serum. Cell suspensions were diluted and, between 6×10^4–1.2×10^5 bone marrow cells, were injected into a caudal vein of each mouse (10 animals per group) that had been exposed to 9.5 Gy 2–3 h earlier. The number of macroscopic colonies per spleen was determined 8 days later.

Hematopoietic progenitor cells committed to granulocyte/macrophage development were assayed as described by Vacek et al. (1991). Bone marrow cells (8×10^4–1.3×10^5) were plated in triplicate in a semisolid environment created by plasma clot, containing Iscove's modification of Dulbecco's medium (IMDM, TechGen Int. Ltd., UK), supplemented with antibiotics (penicillin, 100 U/ml and streptomycin, 1000 μg/ml) and L-glutamine (Calbiochem-Behring, La Jolla, USA) in a concentration of 1.2 mg/ml plus 15–20% newborn calf serum (TechGen Int. Ltd., UK), 10% murine lung-conditioned medium (LCM), 10% citrate bovine plasma and 3% $CaCl_2$ (Biotika, Slovenská Ľupča, Slovakia). The cultures were incubated at 37°C in a fully humidified atmosphere of 5% CO_2 in air for 7 days. Colonies of at least 50 cells were counted at 30 × magnification. The cell suspension used for these assays represented a pool of tissues from 5–7 mice at each time.

The percentage of colony-forming cells (CFC; CFU-S or GM-CFC) in S-phase of the cell cycle was determined by i.p. administration of 1000 mg/kg b.wt. hydroxyurea (Sigma Chemical Co., St. Louis, MO, USA) in saline. Control groups of mice received saline without hydroxyurea at the same time. Ninety minutes later, the bone marrow was assayed for surviving CFC. The number of CFC in bone marrow from hydroxyurea-injected mice was compared with the number in bone marrow from saline-injected mice, and the percentage decrease in CFC after hydroxyurea injection was taken as the percentage of CFC that was in the S-phase of the cell cycle. Statistical analysis was performed using Student's t-test.

2.7. Assay for CSF activity

In separate experiments, blood was collected by cardiac puncture under anesthesia (Pentobarbital inj., Spofa Praha, Czechia) at various times after i.p. injection of 500 μg BV and serum was separated after coagulation at room temperature. The murine serum as a source of CSF was heated to 56°C for 30 min to inactivate complement, and subjected to the CSF assay. Lungs were aseptically removed at similar periods after administration of BV and placed in IMDM supplemented with antibiotics (penicillin, 100 U/ml and streptomycin, 1000 μg/ml). Whole intact lungs were incubated at 37°C in 10% humidified CO_2 in air for 48 h. At the end of incubation, the supernatants were subsequently collected, pooled, filtered (0.2 μm Minisart NML units, Sartorius, Germany) and assayed for CSF activity.

3. Results

The content of CFU-S and GM-CFC per femur was measured in bone marrow from non-irradiated mice 24 h after BV or saline injection (i.e. at the time

of presumed irradiation) (Table 1). Prior to irradiation, the number of day-8 CFU-S and GM-CFC per femur was not significantly different in mice 24 h after injection with 500 μg BV or saline. Simultaneously, a significant diminution in bone marrow cellularity occur at hours 6 ($p<0.05$) and 24 ($p<0.001$). At this time point, cellularity of bone marrow achieved only 83% of the level of the control group (Table 2, Fig. 1). The number of GM-CFC increased significantly at 6 h ($p<0.01$). After that, a decrease to the level of that in the intact control animals followed. In the serum, which was used as an assumed source of CSF, increased CSA did not appear in any of the investigated intervals during 24 h. On the other hand, in the period of the first 3 h after application of BV, CSA decreased in the lungs, whereas in the following period (6–24 h) CSA achieved the level which was observed in the lungs of the intact control animals (Fig. 1).

The hydroxyurea-induced decrease in CFU-S and GM-CFC was used to determine the percentage of CFU-S and GM-CFC that was in the S-phase of the cell cycle. The numbers of CFU-S and GM-CFC in the S-phase were approximately 20% and 38% respectively, for bone marrow cells from control mice. Significant increases in the percentage of CFU-S and GM-CFC (34%, $p<0.05$ and 65%, $p<0.01$ respectively) in the S-phase of the cell cycle were apparent 24 h after administration of BV (Table 1).

Measures of GM-CFC are good indications of myeloid hemopoietic activity in animals recovering from exposure to radiation. After total body sublethal (6 Gy) irradiation, bone marrow cellularity and the number of GM-CFC decreased markedly. In contrast, Table 2 shows that 5, 9 and 14 days, after exposure to 6 Gy, there was evidence of an earlier recovery of bone marrow GM-CFC number and cellularity in mice injected with BV before irradiation. Five, 9 and 14 days after irradiation in saline-injected mice, the number of bone marrow cells was less than 10%, 50% and 86% respectively, and number of GM-CFC was less than 2%, 6% and 10%, respectively, of the number in normal non-irradiated mice. The values at these time points increased 1.5- to 4-fold in bone marrow from mice injected with BV before irradiation.

From the observed changes of the colony-stimulating activity (CSA) of the lungs after the irradiation with the sublethal dose 6 Gy (Fig. 2) results immediately after irradiation, there appears a statistically significant decrease ($p<0.01$) of CSA in the both experimental groups of the animals in comparison with the non-irradiated control groups. Whereas in the comparison with the control (saline) irradiated animals, the decrease of CSA was more significant ($p<0.01$) in the group of mice which administered BV before irradiation. The CSA of the lungs in both groups reached the level of non-irradiated control animals after five days.

It is established that BV induces the synthesis of

Table 1

Decrease in the percentage of colony-forming cells after administration of hydroxyurea to mice 24 h after saline or Broncho-Vaxom®

Survival of colony-forming cells after hydroxyurea injection	Day 8 CFU-S		GM-CFC	
Number per femur ($\times 10^3$)[a]	Saline injection (n)[b]	Broncho-Vaxom® injection (n)[b]	Saline injection (n)[b]	Broncho-Vaxom® injection (n)[b]
Non-injected mice	4.79 ± 1.03 (3)	4.83 ± 0.74 (3)	30.19 ± 0.15 (3)	30.63 ± 3.70 (3)
Injected mice	3.71 ± 0.76 (3)	3.09 ± 0.19 (3)	18.22 ± 1.67 (3)	10.19 ± 1.34 (3)
Colony-forming cells in S-phase (%)	20.48 ± 2.50 (3)	34.33 ± 5.77 (3)[c]	38.11 ± 4.44 (3)	65.88 ± 5.79 (3)[d]

Mice were administered 0.4 ml saline or 500 μg Broncho-Vaxom by i.p. injection 22.5 h prior to administration of hydroxyurea (1000 mg/kg b.wt.) or saline.

[a] Cells were pooled from both femurs of five mice per group in each study 90 min after hydroxyurea injection, values represent the means ± SEM from individual studies.

[b] Number of studies.

[c] $p<0.05$.

[d] $p<0.01$.

Table 2

Recovery of hemopoiesis in bone marrow after 6 Gy of irradiation

Time after irradiation	Irradiated (saline)		Broncho-Vaxom-treated and irradiated	
	Number of cells $\times 10^6$ per femur	Number of GM-CFC per femur	Number of cells $\times 10^6$ per femur	Number of GM-CFC per femur
0 day	21.24 ± 0.39	22030.80 ± 614.10	17.75 ± 0.43[b]	23128.80 ± 503.10
5 days	2.06 ± 0.18	249.79 ± 28.80	7.30 ± 0.81[b]	1148.45 ± 136.50[b]
9 days	10.54 ± 1.58	1280.76 ± 170.50	15.36 ± 0.98[a]	2646.17 ± 328.31[b]
12 days	18.30 ± 1.22	2163.67 ± 297.10	22.53 ± 1.22[a]	4873 ± 441.96[b]

Mice were injected with saline or Broncho-Vaxom® (500 μg per mouse, 24 h prior to irradiation) and exposed to a 6 Gy dose of radiation on day 0. Donor bone marrow cells were pooled from five mice per group for GM-CFC assays (mean ± SEM).
[a] $p < 0.05$.
[b] $p < 0.001$.

prostaglandin E_2 (PGE_2) in vitro (Bottex et al., 1988), which induces radioprotection when applied immediately before irradiation (Walden et al., 1987). We therefore tested the role of PGE_2 in the process of radioprotection induced by BV.

We assessed the effect of the prostaglandin synthesis inhibitor, indomethacin on the radioprotection conferred by BV (Table 3). The survival of mice receiving indomethacin and BV was greater than that for mice receiving BV alone. Administration of indomethacin alone did not promote survival after exposure to this dose of radiation.

4. Discussion

We have previously demonstrated that BV can enhance the survival of mice if administered prior to a 9.5 Gy dose of gamma radiation (Fedoročko et al., 1992; Fedoročko and Brezáni, 1992). Results presented in this report demonstrate that administration of BV significantly promotes the recovery of bone marrow cells and bone marrow GM-CFC in sublethally irradiated mice. Data in this paper and elsewhere (Fedoročko et al., 1992) demonstrate, that compared with saline-injected mice, there were at

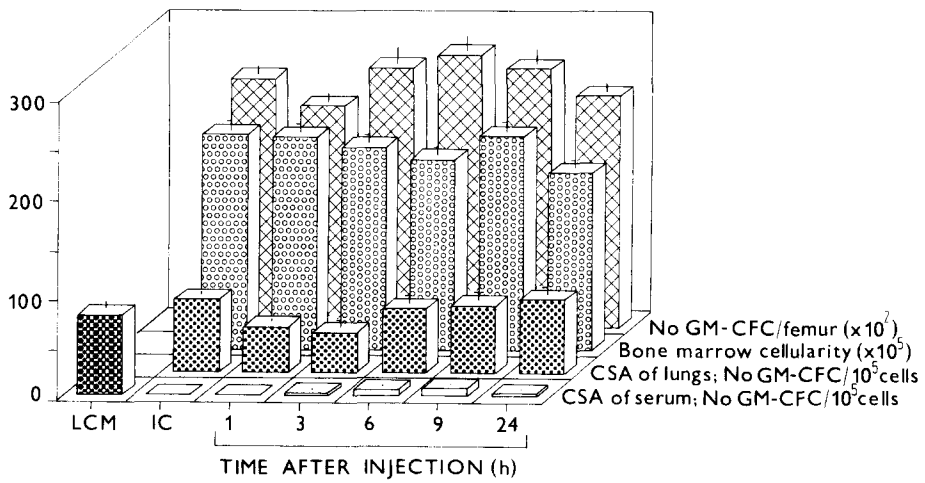

Fig. 1. Effect of Broncho-Vaxom® (500 μg per mouse i.p.) on murine blood serum and lung-conditioned medium colony-stimulating activity, bone marrow cellularity and GM-CFC content in femur. Data represent mean ± SEM from two different experiments of 7–10 mice/group/experiment. IC = intact mice. LCM = lung conditioned medium.

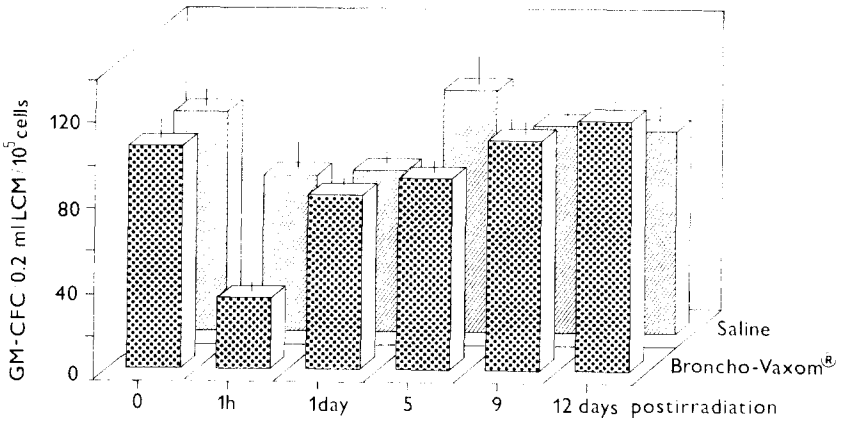

Fig. 2. Effect of Broncho-Vaxom® (500 μg per mouse) on lung-conditioned medium colony-stimulating activity in irradiated mice. Mice were injected i.p. 24 h before exposure to 6 Gy on day 0 and killed on the days indicated. Each point represent mean ± SEM from two different experiments of 5 animals/group/experiment.

least twice at many day-8 CFU-S and GM-CFC per femur in BV-injected mice within 5–14 days after 6 Gy irradiation.

The decrease in radiosensitivity of hemopoietic tissue in immunomodulator pretreated mice has been partly attributed to mobilization of stem cells from bone marrow to spleen, to the release of humoral factors, and to the stimulation and transition of stem cells in to the cell cycle in pre-irradiated animals (Neta, 1988b). In the present studies there was some evidence of cell mobilization after administration of BV. 24 h after BV injection, the number of day-8 CFU-S and GM-CFC per femur was not significantly different from saline pretreated mice. However, bone marrow cellularity was significantly reduced at that time as compared to saline-injected mice. These results, along with the previously reported increase in E-CFU (Fedoročko et al., 1992), demonstrate that some stem cells were mobilized from the bone marrow into circulation after injection of BV, since peripheral blood white cells, plateles and hematocrit values all recovered more quickly in BV-pretreated mice than in radiation control mice (Macková and Fedoročko, 1993).

In the present studies, there is evidence that at the time of irradiation, BV stimulates transition of CFU-S and GM-CFC into the cell cycle. These findings may explain, in part, the radioprotective properties of BV, since the late S-phase of the cell cycle is reported in numerous studies to be the most radioresistant phase of the cell cycle (Sinclair and Morton, 1966; Boggs et al., 1973; Denekamp, 1986). Moreover, radioprotection by agents such as IL-1, Ivastimul and MTP-PE/MLV (Neta et al., 1987; Schwartz et al., 1987; Vacek et al., 1990; Fedoročko, 1994) has been associated with increases in some stem cell compartments in the S-phase of the cell cycle at the time of irradiation. In this context, it is interesting that production of CSF was not detected within the first 24 h following administration of BV, because induction of bone marrow cell cycling and

Table 3

Effect of indomethacin on radioprotection with Broncho-Vaxom® evaluated by mice survival

Group	Survival %	(n)[a]	Mean survival time (days)
(1) Broncho-Vaxom	60% (12/20)	2	–
(2) Broncho-Vaxom + IND	95% (19/20)[b]	2	–
(3) IND	0% (0/20)	2	13.63 ± 0.45
(4) Control (saline)	0% (0/20)	2	12.74 ± 0.40

Groups of 20 mice received 500 μg of Broncho-Vaxom i.p. (1), 500 μg of Broncho-Vaxom + 2 × i.m. injections of 40 μg of indomethacin (IND) at 24 and 3 h prior to irradiation (2), indomethacin alone (3), or saline (4). Twenty four h following Broncho-Vaxom or saline injections mice were irradiated with 9.5 Gy.

[a] Number of studies.

[b] $p < 0.05$ (as compared to Broncho Vaxom alone).

cell proliferation is associated with secretion of colony-stimulating factors (Broxmeyer et al., 1987; Metcalf, 1986). No evidence exists that BV can induce CSF in cells.

The ability of BV to induce the secretion of IL-1 may indicate that it is able to accelerate the restoration of functional hemopoietic cells, because IL-1 has recently been reported to be a radioprotective agent (Neta et al., 1986a,b; Schwartz et al., 1988, 1989). Another recognized activity of BV, that may contribute to its radioprotective effect, is the enhancement by BV of the release of prostaglandins, which exhibit a radioprotective effect when administered immediately before irradiation (Walden et al., 1987). In vitro studies showed an increased IL-1 production with a maximum within the first 24 h, and prostaglandin production after 24 h culture following BV activation of a macrophage cell-line (Bottex et al., 1988). If this occurs also in vivo, the previous finding of a maximal effect of BV at 24 h could coincide with the time of the maximal IL-1 level and intensive production of prostaglandins, which may act as radioprotective substances. We, therefore, assessed the effect of indomethacin, an inhibitor of prostaglandin synthesis, on the radioprotection conferred by BV. Simultaneous administration of BV and indomethacin to mice resulted in an additional antiradiation effect. Based on the results of these survival studies, the radioprotection induced with BV is not prostaglandin-mediated.

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