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Radioprotection of mice by the bacterial extract Broncho-Vaxom^R: haemopoietic stem cells and survival enhancement

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Abstract. Pretreatment of mice with 50-1000 μg of the bacterial extract Broncho-Vaxom^R (BV, free of endotoxin) before sublethal irradiation induced an increase in the number of endogenous haemopoietic stem cells (E-CFU). The degree of radioprotection was dependent on both the time of administration and the dose of BV. An optimal E-CFU survival was observed when 500 μg of BV was administered i.p. 24 h before irradiation.

BV did not affect the day 9 CFU-S survival in the bone marrow directly after irradiation. However, 5, 9 and 12 days after irradiation, the number of day 9 CFU-S was almost 2-fold higher in the bone marrow of BV injected mice. Pretreatment with BV protected C57Bl/6 mice in a dose-dependent manner from the lethal effect of ionizing radiation. A single dose (50, 100, 250, or 500 μg) of bacterial lysate injected i.p. 24 h before 9·5 Gy γ-rays ($LD_{100/21}$) protectd 16%, 25%, 80%, and 94% of C57Bl/6 mice, respectively. The dose reduction factor in the case when the BV (500 μg per mouse) was administered at that time was 1·18 (95% CL 1·12, 1·25).

1. Introduction

It has been recognized that many agents capable of non-specifically enhancing immunological and haemopoietic responses can also function as radioprotectants (Patchen *et al.* 1988). For example, administration of immunomodulatory substances such as bacterial lipopolysaccharide, glucan, BCG and Ivastimul to mice, prior to their exposure to ionizing radiation, results in an increase in the number of mice that survive beyond 30 days (Behling 1983, Patchen *et al.* 1988, Vacek *et al.* 1990).

Broncho-Vaxom^R (BV), a lyophilized fractionated alkaline bacterial extract, is used as a polyvalent immunobiotherapeutic agent active in the treatment of respiratory tract infections, particularly acute and chronic bronchitis (Maestroni and Losa 1984, Palma-Carlos *et al.* 1987, Heintz *et al.* 1989).

The mechanism of action of BV is not yet fully understood. Experimental studies indicate that it enhances immune responses, both cellular (Clot and Andary 1980, Emmerich *et al.* 1990) and humoral (Bosch *et al.* 1983, Emmerich *et al.* 1990). Treatment of mice enhanced 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 *in vitro* (Bottex *et al.* 1988). BV can strongly potentiate metabolic and functional activities of murine macrophages in synergism with macrophage-activating factors leading to the acquisition of cytotoxic properties *in vitro* against tumour cells and intracellular microbes. This effect appears to be independent of residual contamination by endotoxin (Mauel *et al.* 1989).

In the present study we have attempted to verify the presumptive radioprotective effect of BV using the haemopoietic stem cell and animal survival assays.

2. Materials and methods

2.1. *Mice*

Female C57Bl/6 mice, 8-10 weeks old, were obtained from Velaz (Prague, CSFR). Animals were quarantined for a period of 2 weeks. They were housed in rodent cages, five to seven animals per cage at about 23°C, and they were given LD-food (Velaz, Prague) and tap water acidified to pH 2·4 *ad libitum*. Research was conducted according to principles enunciated in the 'Guide for the Care and Use of Laboratory Animals', prepared by the State Veterinary Office of Slovak Republic, Bratislava.

2.2. *Treatment with Broncho-Vaxom^R*

BV (Biogal, Debrecen, Hungary, under licence of OM Laboratoires, Geneva, Switzerland) is a lyophi-

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lized fractionated alkaline extract of the eight most common bacteria of the upper respiratory tract (*Diplococcus pneumoniae, Haemophilus influenzae, Klebsiella ozaneae, Klebsiella pneumoniae, Neisseria catarrhalis, Staphylococcus aureus, Streptococcus pyogenes* and *Streptococcus viridans*), free of endotoxins (less than 0·0002% by *Limulus* and pyrogenicity tests, Bottex *et al.* 1988). The final dry preparation contains (by weight) 35% protein, 8% free amino acids, 10% lipids, 8% nucleotides, 2% carbohydrates and approximately 37% salts (Mauel *et al.* 1989). Immediately before use the drug was resuspended in saline in a volume of 0·2 ml (50 or 100 μg) or 0·4 ml (250, 500 or 1000 μg) and administered i.p. Control animals were administered 0·2 ml or 0·4 ml saline at the same time.

2.3. *Irradiation*

Mice were placed in Plexiglas containers and exposed to 1–10·5 Gy (haemopoietic stem cell assay) or to 8–11·5 Gy (survival assay) of γ-rays (0·3 Gy/min) at different time intervals after or before BV injection. A Chisostat ^{60}Co source (Chirana, CSFR) was used for all irradiations.

2.4. *Haemopoietic stem cell assay*

Endogenous colony-forming unit (E-CFU) formation was assayed by the method of Till and McCulloch (1963). Briefly, mice received 6–10·5 Gy of radiation. They were killed by cervical dislocation at 10 days postirradiation and their spleens were removed and fixed in Bouin's solution. The number of macroscopic colonies per spleen was determined, and the mean and its standard error were calculated. The dose reduction factor (DRF) was calculated on the basis of a logarithmic transformation of the means and the standard errors according to Smith *et al.* (1966).

The survival of haemopoietic stem cells following increasing doses of radiation and recovery of haemopoiesis in bone marrow after 6 Gy were determined by the exogenous spleen colony (CFU-S) assay (Till and McCulloch 1961). Groups of mice ($n=5$) were administered i.p. either 500 μg of BV/mouse or saline alone 24 h prior to receiving 0, 1, 2, 3 or 5 Gy. Immediately after irradiation (survival CFU-S) or 5, 9 and 12 days after irradiation (CFU-S recovery) both femora were removed, and a bone marrow cell suspension was made. Depending on the radiation dose given to the donor mice, between 5×10^4 and 1×10^6 bone marrow cells were injected i.v. into mice ($n=10$/group) that had been exposed to 9·5 Gy 2 h earlier. The number of macroscopic colonies per spleen was determined 9 days later. Statistical analysis was performed using Student's *t*-test.

2.5. *Survival assay*

Survival was monitored daily and was reported as the percentage of animals surviving 30 days after

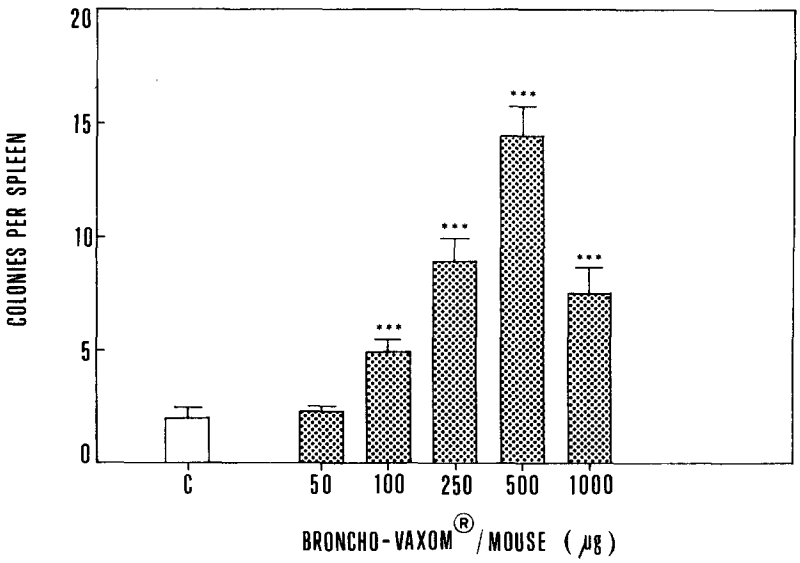

Figure 1. Effect of Broncho-Vaxom^R on the number of endogenous spleen colonies (E-CFU). Mice were injected i.p. 24 h before irradiation with 8 Gy. Data represent the means ± SEM from three experiments and each data point represents 23–33 mice. C = control (irradiated mice); *** = $p < 0·0001$ (as compared to control).

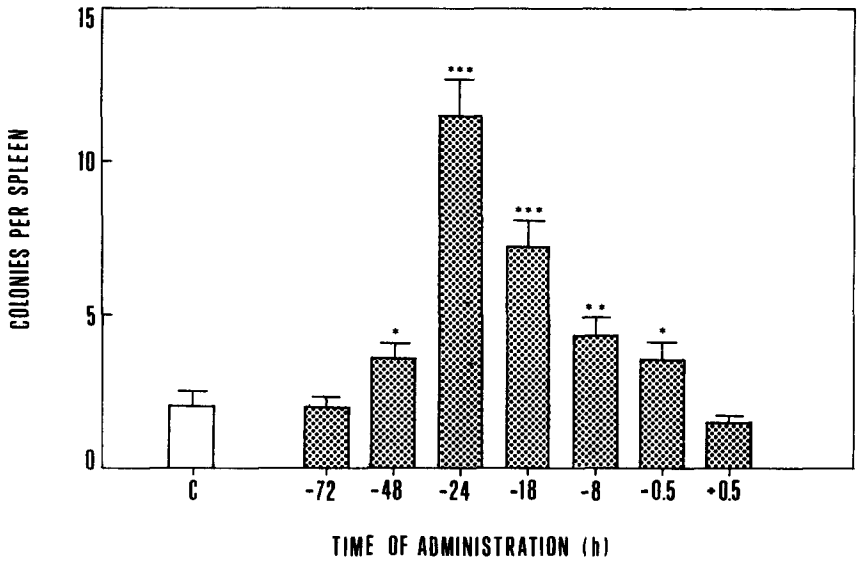

Figure 2. Time dependence of radioprotection on E-CFU by Broncho-Vaxom^{R} 500 μg per mouse injected i.p. at the hours indicated before (−) or after (+) 8 Gy whole-body irradiation. Data represent the means ± SEM from three experiments and each data point represents 18–25 mice. C=control (irradiated mice); *=$p<0{\cdot}05$; **=$p<0{\cdot}001$; ***=$p<0{\cdot}0001$ (as compared to control).

irradiation. Each treatment group within each experiment consisted of 10–15 mice. The dying animals in this experiment were killed when moribund. On day 31, surviving mice were euthanized by cervical dislocation. Experiments were repeated two to five times. The DRF was calculated by dividing the treament $LD_{50/30}$ by the control $LD_{50/30}$.

3. Results

Pretreatment of mice with BV induced a drug dose-dependent protection of E-CFU following exposure to sublethal radiation (Figure 1). The highest sparing of E-CFU was found in mice that were injected with 500 μg of BV (14·4 ± 1·33 E-CFU; $n=30$), which gave a 7-fold increase over control-

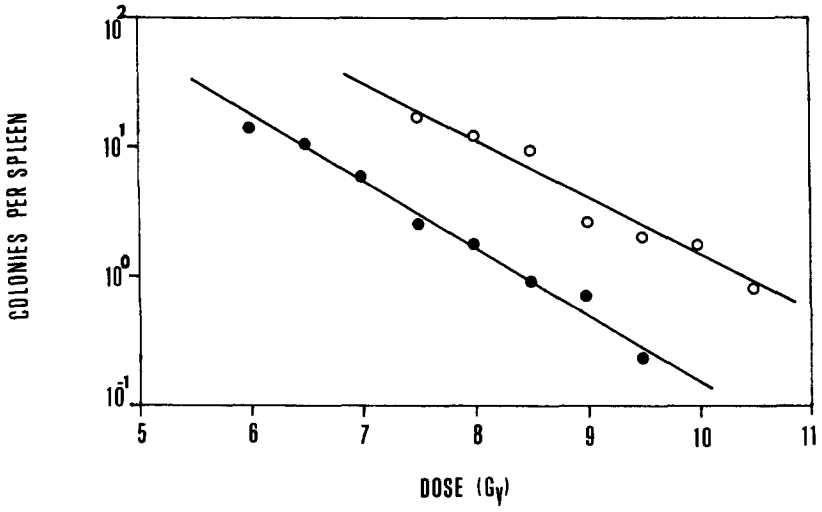

Figure 3. Protective effect of 500 μg Broncho-Vaxom^{R} per mouse on endogenous spleen colony formation (E-CFU) in mice receiving graded doses of radiation. BV-treated mice (○) and control mice (●). Each data point represents 16–30 mice. Treatment effect was compared at the levels of 10 E-CFU/spleen (BV versus saline, $p<0{\cdot}001$). The lines were fitted by least-squares regression analysis.

Table 1. Survival of day-9 CFU-S 0·5 h after irradiation of saline or Broncho-Vaxom^R injected mice

Radiation dose (Gy)	Day-9 CFU-S/femur ($\times 10^3$)[a]	
	Irradiated (saline)	Broncho-Vaxom^R-treated and irradiated
0	3·16±0·94	3·03±0·55
1	1·29±0·13	1·21±0·37
2	0·2±0·13	0·31±0·16
3	0·13±0·007	0·04±0·02
5	0·02±0·005	0·01±0·003

Mice were administered 500 μg of Broncho-Vaxom^R 24 h prior to their irradiation. Thirty minutes after irradiation, cells from both femurs of five mice were pooled per group.

[a]CFU-S/femur were calculated from mean±SEM of the number of colonies from ten spleen per group.

injected mice (2·04±0·44 E-CFU; $n=35$). Figure 2 shows that the time of injection of BV was a critical factor in the expression of E-CFU. The highest augmentation in E-CFU number was reached with bacterial lysate administered 8–24 h before irradiation. The optimum time administration was 24 h before irradiation. Postirradiation injection was ineffective. Figure 3 shows the dependence of the number of E-CFU on the radiation dose in control and BV-treated mice. In both groups the number of colonies decreased as the dose of radiation increased. However, the number of spleen colonies was higher in the BV-treated mice (for doses 7·5–9·5 Gy, BV versus saline, $p<0·001$). For example, the calculated radiation dose which resulted in an average of 10 E-CFU per spleen for BV-treated mice was significantly greater than that for controls (8·09±0·03 Gy versus 6·60±0·03 Gy, $p<0·001$). The DRF resulting from these values was 1·240±0·005 (SE).

The number of day-9 CFU-S surviving was determined 0·5 h after irradiation of saline or BV-injected mice, and there was no significant difference (Table 1). Table 2 shows that 5, 9 and 12 days after 6 Gy the number of day-9 CFU-S was 2-fold higher in the bone marrow from BV-injected mice than from saline-treated animals. Bone marrow cellularity, as well as the number of CFU-S in bone marrow after irradiation, demonstrates that BV injection prior to irradiation accelerated the haemopoietic recovery in sublethally irradiated mice.

The optimal dosage and time for the preirradiation administration of BV, which enhances animal survival, is shown in Figures 4 and 5. At 24 h prior to irradiation, mice were injected i.p. with bacterial extract in doses ranging from 50 to 500 μg per mouse. This time interval has been proven to be an effective administration schedule for most of the immunomodulators (Patchen *et al.* 1988, Vacek *et al.* 1990). From Figure 4 it appears that 16% of the mice pretreated with 50 μg per mouse BV survived 30 days after 9·5 Gy, and 25%, 80% and 94% survived when pretreated with 100, 250 or 500 μg per mouse BV, respectively. The optimal time lapse between irradiation and administration of the drug was determined by injecting 500 μg per mouse either at 0·5, 8, 18, 24, 48 or 72 h before, or 0·5 h after irradiation with 9·5 Gy. As shown in Figure 5, protection from 9·5 Gy was optimal when the drug was given 0·5–48 h before irradiation. Maximal radioprotection was obtained when BV (500 μg per mouse) was administered 24 h before irradiation. The $LD_{50/30}$ for BV-pretreated mice was 9·81 Gy (95% CL 9·51, 10·12). This was significantly greater ($p<0·001$) than the value of 8·32 Gy (7·96, 8·68) calculated for the saline control. For C57Bl/6 mice irradiated at 24 h after i.p. injection of 500 μg per mouse BV, the DRF was 1·18 (1·12, 1·25) (Figure 6).

4. Discussion

The results show that BV is capable of protecting haemopoietic tissue of mice against radiation injury, both in terms of haemopoietic stem cell and mouse survival. The DRF of 1·18 for $LD_{50/30}$ is comparable

Table 2. Recovery of haemopoiesis in bone marrow after 6 Gy of radiation

Time after irradiation	Irradiated (saline)		Broncho-Vaxom^R-treated and irradiated	
	Number of cells $\times 10^6$ per femur	Number of CFU-S per femur	Number of cells $\times 10^6$ per femur	Number of CFU-S per femur
5 days	2·13±0·26	16·27±4·5	4·67±0·81[b]	46·88±12·5[a]
9 days	7·98±0·21	202·93±64·7	11·72±0·59[b]	437·22±96·8
12 days	9·49±0·84	288·10±11·7	14·70±0·93[b]	665·37±235·1

Donor bone marrow cells were pooled from five mice per group for CFU-S assay (mean±SEM).
[a]$p<0·05$. [b]$p<0·01$.

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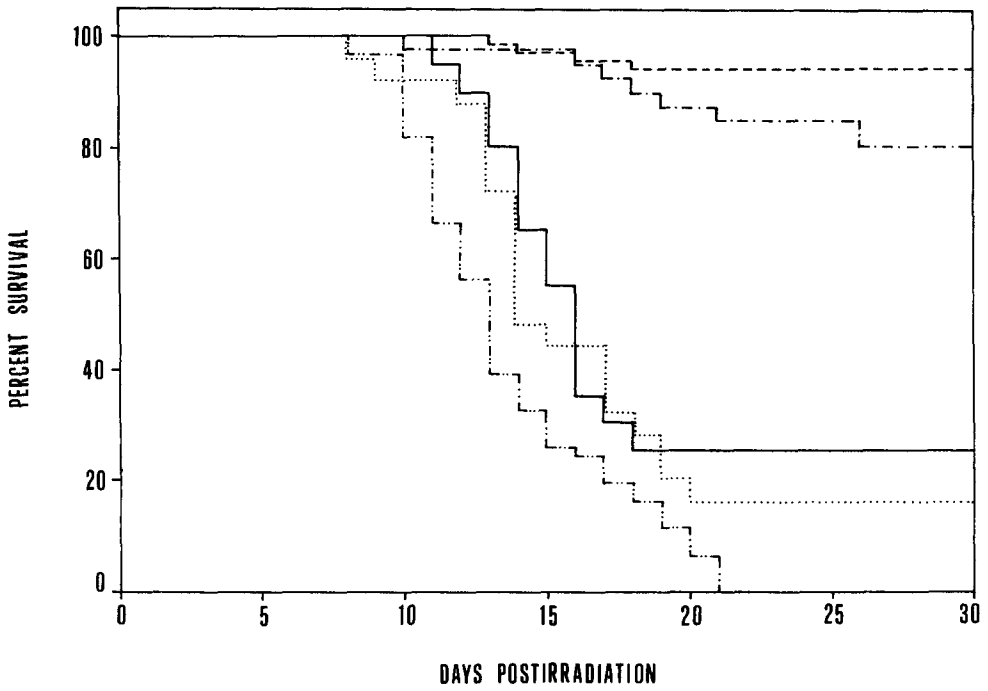

Figure 4. Effect of graded doses of Broncho-Vaxom^{R} on the 30 day-survival of 9·5 Gy irradiated mice, injected i.p. 24 h before irradiation. Data represent the mean of two to five separate experiments. Control (–··–··–), $n=62$; 50 μg (·····), $n=25$; 100 μg (——), $n=20$; 250 μg (–·–·–), $n=40$; 500 μg (– – –), $n=70$.

to the DRF reported for other radioprotective immunomodulators (Neta *et al.* 1986b, 1988b, Wu *et al.* 1989, Patchen *et al.* 1990). The wide range of its radioprotective action (Figure 5) is uncommon even for radioprotectors of an immunomodulatory nature. Though a 20–24 h time interval between administration and irradiation appears to be the most effective administration schedule for the majority of radioprotective immunomodulators, several exceptions have been reported. Patchen *et al.* (1988)

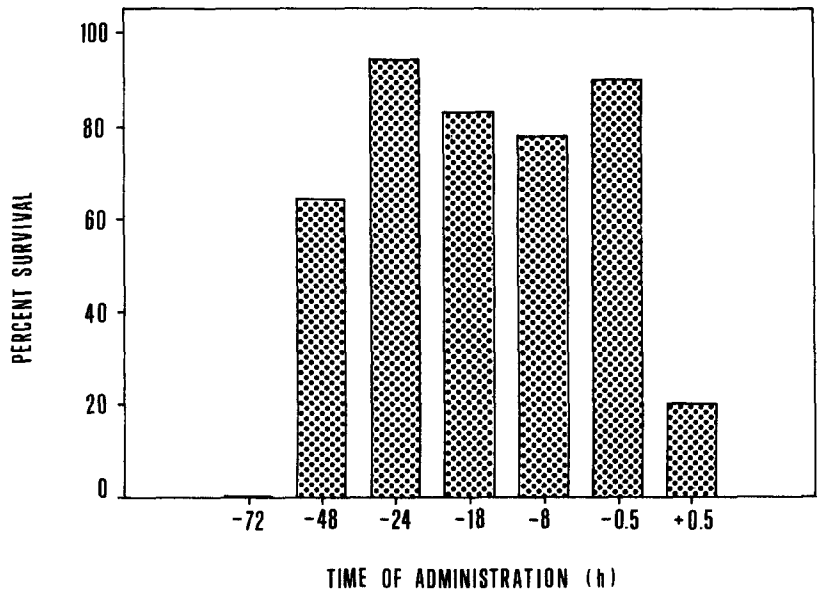

Figure 5. Thirty-day survival of 9·5 Gy-irradiated mice as a function of the time at which 500 μg of Broncho-Vaxom^{R} was administered. Data represent the mean of three to five separate experiments and each data point represents 30–70 mice.

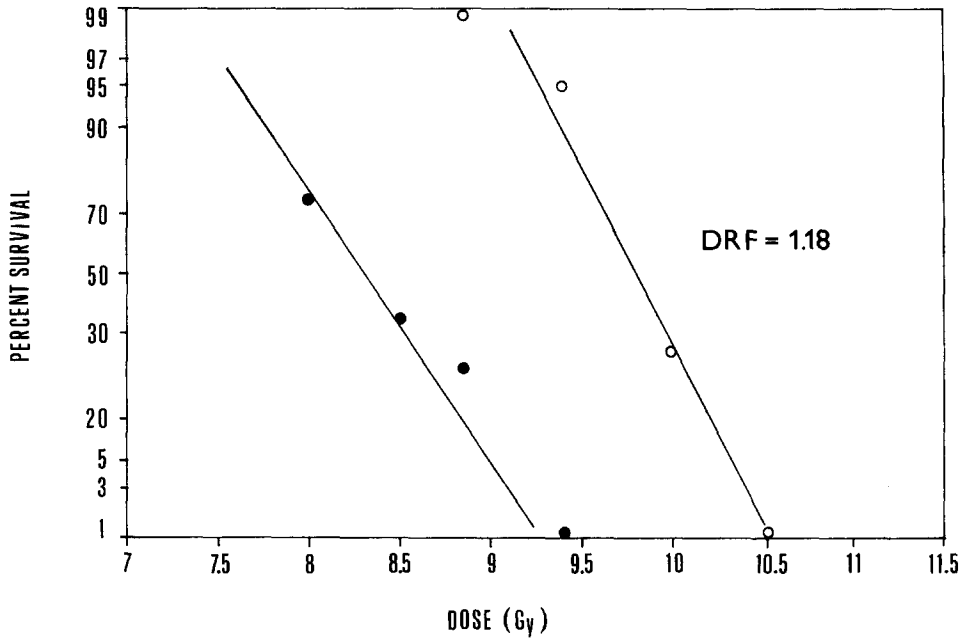

Figure 6. Animal survival as a function of radiation dose for mice injected with Broncho-Vaxom^{R} (500 μg per mouse, ○) or saline (●) 24 h before irradiation. DRF = LD_{50} (BV + irradiation)/LD_{50}(saline + irradiation), using probit analysis. Each data point represents 20–25 mice. Based on $LD_{50/30}$ values: BV versus saline, $p < 0{\cdot}01$.

found that Imuthiol administered 20–24 h before irradiation enhanced haemopoietic recovery but had no effect on the survival of animals. However, if given 15–30 min before exposure Imuthiol had a positive influence on both responses. Additionally, one of the immunomodulators reported by Maisin *et al.* (1986) produced a 60% increase in LD_{50} when given 18 h before irradiation, and an 80% increase when given 15 min before. In our experiments a maximum radioprotective effect was achieved using a time interval of 24 h, but a relatively high percentage of mice survival was also observed when administered 30 min before irradiation. This indicates that various mechanisms and endogenous mediators contribute to the radiation protection observed in BV-treated mice.

Within days 5–12 after 6 Gy there were 2-fold more CFU-S per femur in BV-injected than in saline-injected mice. However, as shown in Table 1, BV-treatment did not cause a significant change in the response of bone marrow stem cells 0·5 h after irradiation. In addition there was no significant difference in the number of day-9 CFU-S from non-irradiated mice 24 h after saline or BV injections (Table 1). Similar results have been reported after sublethal irradiation in endotoxin or IL-1-injected mice (Smith *et al.* 1969, Schwartz *et al.* 1988, 1989). In those studies the number of day-12 CFU-S was 2-fold higher between days 5 and 12 after IL-1 injection. Also, the recovery of CFU-S in endotoxin-injected mice was approximately 2 days earlier than in control mice, and was not associated with an increase in the number of CFU-S that survived irradiation.

The mechanism(s) of BV radioprotection remains unknown. The multiplicity of biological effects of this bacterial extract provides a number of possible mechanisms of radioprotection. It was observed by Mauel *et al.* (1989) and Bottex *et al.* (1988), that BV is a macrophage activator. Macrophages are one of the primary cellular targets of inflammatory and immunoenhancing agents that are radioprotective for mice (Richardson and Alving 1987, Patchen *et al.* 1988, Vacek *et al.* 1990). Patchen *et al.* (1988) have previously assumed that the majority of immunomodulators 'affecting macrophages may mediate their radioprotective effects not only by direct macrophage activating and enhancing the nonspecific host defense mechanism but also by inducing the release of cytokines capable of either directly or indirectly enhancing additional hemopoietic and immunologic activities'. Therefore, one of the possible ways of radioprotection is BV-induced secretion of IL-1 and prostaglandins. Recently, Neta *et al.* (1986a,b, 1988a) and many others showed that IL-1 was a radioprotectant under both *in vivo* (Morrissey *et al.* 1988, Wu *et al.* 1989, Schwartz *et al.* 1988, 1989) and *in vitro* (Gallicchio *et al.* 1989) conditions. Previous studies demonstrated that IL-1 injection before irradiation increased the number of E-CFU in irra-

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diated mice (Neta *et al.* 1986b). The same is true for prostaglandin E_2, which exhibits a radioprotective effect when administered 5–60 min before irradiation in doses of 1–40 μg per mouse (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 administration (Bottex *et al.* 1988). If this occurs also *in vivo* the present finding of a maximal effect 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 in the organism.

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