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Radioprotective effects of the immunostimulating lauroylpeptide LtriP (RP 56142)

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Abstract. The lipopeptide lauroyl-L-Ala-γ-D-Glu-L,L-A_2pm (LtriP) increased the resistance of mice to the lethal effect of γ-ray irradiation. The radioprotective effect was dependent on the doses of LtriP and of radiation. Maximum survival was observed when the lipopeptide was injected on two successive days before irradiation. This activity seems to be related to immunostimulating functions, since the non-immunostimulating analog lauroyl-L-Ala-γ-D-Glu-D,D-A_2pm-Gly, containing D,D-diaminopimelic acid, was not radioprotective. The protective activity might result from an induction of cytokines, such as IL-1, TNF and M-CSF, since LtriP induced the mRNA expression and the secretion of these immunomodulators.
Key words. Lipopeptides; immunostimulation; irradiation; M-CSF.

A variety of immunopotentiating agents (e.g., BCG and *Corynebacterium parvum*[1], endotoxins[2], glucan[3], leukotriene[4]) protects mice from the effect of lethal γ-ray treatment when administered before irradiation. It has been suggested that this radioprotective effect is due to the increased synthesis of immunological factors, such as cytokines and hemopoietic growth factors, which interact to confer radioprotection[5]. Free radical scavengers are another type of compounds that may have this activity; by reacting with free radical products generated during ionizing irradiation[6], they may inhibit detrimental processes mediated by free radicals, such as lipid peroxidation[7].

Lauroylpeptides were the first immunostimulating lipopeptides to be described[8]. Recently, two such compounds, lauroyl-L-Ala-γ-D-Glu-L,L-A_2pmNH_2-Gly(LtetraP L, LA_2pm, RP 44102) and lauroyl-L-Ala-γ-D-Glu-L,L-A_2pmNH_2 (LtriP, RP 56142), were found to decrease the amount of hepatic microsomal cytochrome P-450 and the level of CCl_4-induced lipid peroxidation[9]. These effects, however, were not observed with lauroyl-L-Ala-γ-D-Glu-D,D-A_2pmNH_2-Gly(LtetraP DDA_2pm, RP 53204). This compound differs from LtetraP L,LA_2pm only in the configuration of the two chiral carbons of diaminopimelic acid (A_2pm), and it is not an immunostimulating agent. This paper describes the effects of the lauroylpeptide LtriP on mice irradiated with γ-rays at lethal doses. As we had previously established the links between immunostimulation, decrease of hepatic induced lipid peroxidation and modulation of hepatic cytochrome P-450[9], here we explored additionally the relationship between immunopotentiation and radioprotection by comparing LtriP with the non-immunostimulating compound LtetraP D,DA_2pm.

Materials and methods

Reagents. The lauroyltripeptide LtriP (RP56142) and the lauroyltetrapeptide LtetraP D,DA_2pm (RP 53204) were synthesized by Rhône-Poulenc Santé laboratories (Vitry sur Seine, France). Other reagents were obtained from the following sources: [5′-^{3}H]-thymidine (185–740 GBq/mmol) and Hybond-N membranes: Amersham (Les Ulis, France); L-glutamine. Boehringer Mannheim (Germany); lipopolysaccharide (LPS) from *Escherichia coli*: Difco (Detroit, USA); sodium pyruvate, FCS and Trypan blue: Flow Laboratories (Irvine, Scotland); penicillin-streptomycin, RPMI 1640 and McCoy medium 25 mM Hepes: Gibco Laboratories (Saisley, Scotland); Concanavalin A (Con A): IBF (Villeneuve-la-Garenne, France); monoclonal IgG anti M-CSF: OSI (Paris, France); lipopolysaccharide (LPS) from *Salmonella enteritidis* and (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) (MTT): Sigma (St. Louis, USA). All other reagents were obtained from Prolabo Laboratories (Vitry sur Seine, France).

Animals. 8–12-week-old (22–30 g) female NMRI, $B_6D_2F_1$ and OF_1 mice were obtained from Iffa-Credo (St. Germain L'Arbresle, France).

Treatment of animals. NMRI mice were injected daily intraperitoneally (i.p.) over various periods of time with different doses of lipopeptides dissolved in saline. Control mice received an equal volume of saline (0.5 ml). Mice were identified individually and controls and LtriP-treated animals were housed in the same cages.

Irradiation. 12 animals per group were irradiated as follows: groups of two mice were placed in a plexiglass container and exposed to a ^{60}Co γ-ray source, such that

they received total body irradiation at various doses. The mean dose rate was 1.4–1.8 Gy/min. The number of surviving animals was recorded daily for 30 days. Under our conditions, when the hemopoietic syndrome was fatal, mice died before 23 days had elapsed.

Hematological analysis. Hematological analyses were performed 1, 8, 12, 15, 18 and 30 days after irradiation. Animals (5 mice at each time) were exsanguinated for blood cell counts. Total white blood cell (WBC) and differential cell counts were performed with an automated Coulter S counter and a Hematrack 590.

Histology. Spleen, thymus, lymph nodes and bone marrow were collected at different times and fixed in buffered neutral 10% formalin. Tissues were trimmed, embedded in paraffin, sectioned into 3-µm slices and stained with hematein-eosin. They were then evaluated for histopathological changes.

In vitro studies

Northern blot analysis. Peritoneal macrophages ($3-6\times10^6$ cells) from $B_6D_2F_1$ mice were incubated with or without LtriP (10 µM) or LPS (*E. coli*, 100 ng/ml) for 6 h[10]. Total mRNA was isolated[11] and analyzed by electrophoresis in 1.2% agarose gels containing 2.3 M formaldehyde in 40 mM 3-(N-morpholine)-propanesulfonic acid buffer, pH 7.0. The mRNA was transferred to Hybond-N membranes and hybridized with ^{32}P-labeled murine probes consisting of the cDNA of IL-1α (residues 415–485), IL-1β (residues 401–471), TNFα (residues 300–369) and M-CSF (residues 259–338), at 42 °C, overnight. Autoradiography was carried out for 5 days with Kodak Xomat AR-5 films[12].

Bone marrow cell (BMC) and spleen cell (SC) proliferation assays. Mice were sacrificed by cervical dislocation and their femurs and spleens were excised. BMC of several animals were pooled, as were SC. Cells were counted in the presence of Turk blue and viable cell number was determined by trypan blue dye exclusion.

BMC proliferation was studied by counting cells present in the serum of OF_1 mice treated or not treated with LtriP[13]. Briefly, BMC were flushed from femurs of mice (5 per group) under sterile conditions. The whole preparation was done at 4 °C; cells were kept in cold McCoy medium supplemented with 1% L-glutamine (2 mM), 1% penicillin-streptomycin (50 U/ml-50 µg/ml) and 2% foetal calf serum (FCS). BMC were dispersed through a needle, filtered through sterile gauze to remove aggregates and debris, washed once with culture medium and counted. The cell suspension (10^5 cells/well) was incubated in quadruplicate with 2% normal mouse serum or 2% serum of OF_1 mice that had been subcutaneously injected 6 h previously with LtriP. When specified, rat anti-M-CSF antibodies or control rat IgG were added to culture.

SC proliferation was evaluated after sub-optimal Con A stimulation. Spleens of NMRI mice (5 per group) were minced in RPMI 1640 medium supplemented with 10^{-5} M 2-mercaptoethanol, 1% L-glutamine, 1% penicillin-streptomycin and 5% FCS. After centrifugation (10 min at 400 × g), red blood cells were removed by hypotonic lysis by adding Gey's solution[14] and leaving them for 5 min at 4 °C. The SC were then washed in culture medium. After centrifugation, SC were resuspended in culture medium, dispensed in triplicate (10^5 cells/well) and cultured with Con A (2.5 µg/ml).

BMC and SC were incubated for 3 days at 37 °C in a humidified 6% CO_2 atmosphere. During the last 6 h, 0.5 µCi/well of ^{3}H-thymidine was added and incorporation was measured by β liquid scintillation. Alternatively, the MTT coloration method of Mosmann[15], as modified by Denizot et al.[16], was used.

Statistics. Proliferative cell responses were analyzed statistically by means of Student's t-test. Survival was evaluated statistically according to the 'Logrank test'[17].

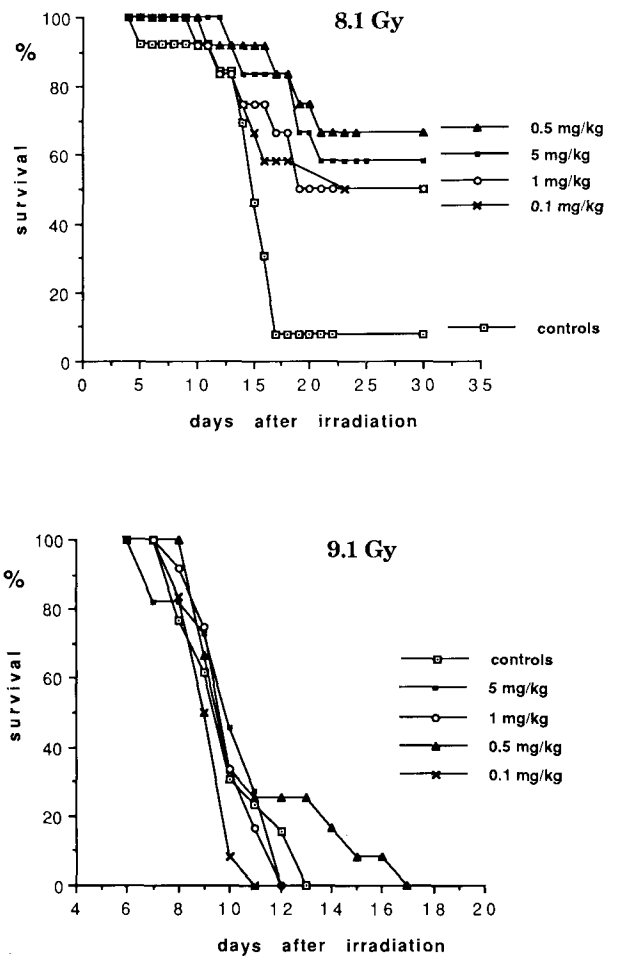

Figure 1. Survival of NMRI mice treated i.p. on 3 successive days before irradiation with different doses of LtriP or saline (controls). Number of animals per group, n = 12.

Results

Effect on survival

Under our experimental conditions, on irradiation of 8.1 Gy resulted in 90% mortality of NMRI mice. The 50% lethal dose (LD_{50}) was established to be around 7.9 Gy. Death occurred between day 8 and day 22.

LtriP, injected before γ-ray treatment, protected mice from mortality due to irradiation. This radioprotection was dependent on the dose of LtriP. With 8.1 Gy, the optimal dose was found to be 0.5 mg/kg (fig. 1); at this dose, 70% of the LtriP-treated animals survived compared to 10% in the untreated group. With 9.1 Gy, a lethal radiation dose after 17 days whatever the LtriP treatment, the longest survival time (16 days) was also obtained with 0.5 mg/kg (fig. 1).

A maximal effect was obtained when LtriP was injected on 2 consecutive days before irradiation (fig. 2). When it was injected 1 day before irradiation, protection was not significant (fig. 2a). Under these conditions *S. enteritidis* LPS gave 90% protection (fig. 2b). The radioprotective effect of LtriP (0.5 mg/kg) was also dependent on the γ-ray dose (fig. 3): 90% survival of mice was obtained for 7.95 Gy, 30% for 8.7 Gy and no survival for 9.1 Gy. The non-immunostimulating lipopeptide LtetraP D,DA_2pm, given under the same conditions at 0.5 mg/kg, was devoid of any radioprotective activity (fig. 4).

Effect on peripheral cell blood counts

The leukocytes, neutrophils, lymphocytes and monocytes of untreated and LtriP (0.5 mg/kg)-treated animals were counted at different times following 7.95 Gy irradiation. In the irradiated groups, the total blood leukocyte count decreased markedly on day 1 and, for surviving animals, returned to the normal level, i.e. that of non-irradiated animals, by day 18 (table). For irradiated LtriP-treated mice, 1 day after irradiation, lymphocyte counts were significantly higher than those of controls, but they decreased to control level at day 8; a slightly accelerated recovery of neutrophils was observed from day 8 on, reaching a level significantly different from that of untreated mice on day 15. No effect was observed on other blood cell parameters, such as the number of monocytes, platelets and erythrocytes (data not shown).

Histological analysis

Following irradiation (7.95 Gy), complete atrophy with signs of severe aplasia was observed in all the lymphoid organs analyzed. In control animals, signs of regenera-

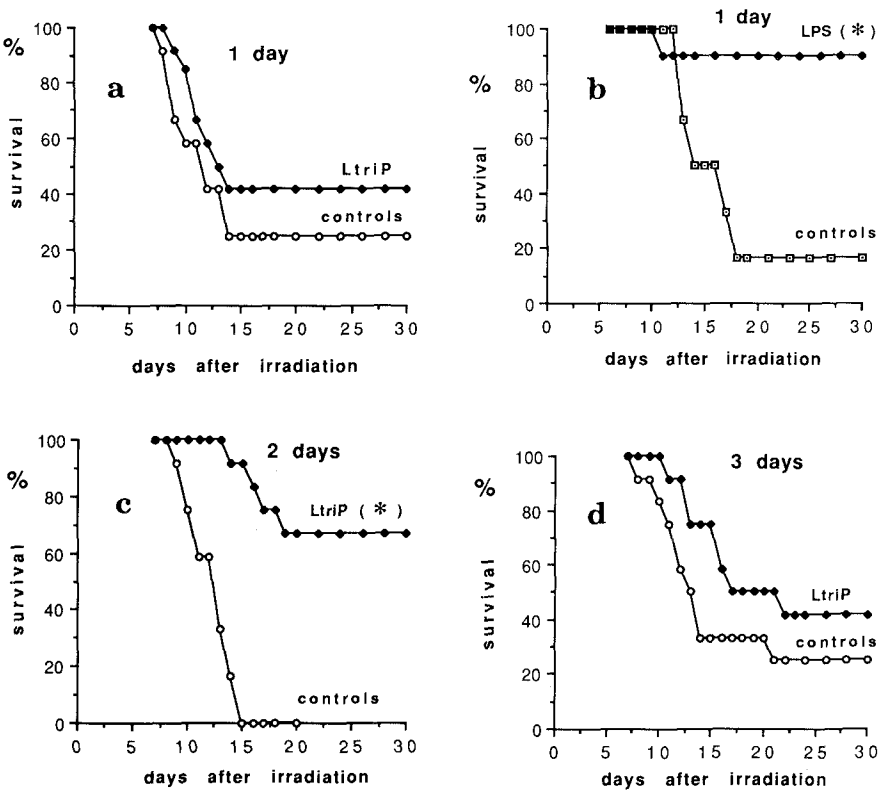

Figure 2. Survival of NMRI mice treated i.p. 1, 2 or 3 days before irradiation (8.1 Gy) with LtriP (0.5 mg/kg), LPS (2 µg/animal) or saline, n = 12.

*Significantly different from control values (Logrank test, $KHI_2 > 3.84$).

tion began to appear on day 8 (appearance of immature cells); in surviving animals, the appearance of bone marrow and spleen was normal from day 18 on, but the thymus still appeared abnormal on day 30.

Administration of LtriP (0.5 mg/kg) did not prevent severe aplasia from occurring on day 1; the condition was similar to that observed in the control group. However, on day 8 bone marrow, spleen and thymus in the LtriP-treated mice displayed an accelerated recovery. On day 15, bone marrow and thymus appeared almost normal in the LtriP-treated group, while signs of regeneration were still observed in the control group. On day 18, regeneration was complete in all organs analyzed, including the thymus.

Histological analysis of the treated and untreated non-irradiated groups showed that LtriP treatment alone had no effect on normal hemopoietic tissues.

In vitro studies

Incubation of murine peritoneal macrophages with LtriP induced the synthesis of IL-1α, IL-1β, TNFα and M-CSF mRNA (fig. 5).

Characterization of M-CSF production in the serum of LtriP-treated mice: a colony-stimulating activity is found in the serum of mice treated with LtriP. The maximal activity is observed 6–8 h after LtriP injection, and levels of CSF return to normal at 24 h[13]. In the present study, this activity was found to be due mainly to M-CSF, as BMC proliferation in the serum of LtriP-treated mice was nearly completely inhibited by anti-M-CSF antibodies (fig. 6).

BMC proliferation in the presence of mouse serum containing M-CSF was also measured. In the absence of irradiation, BMC from LtriP-treated and untreated mice were equally responsive to M-CSF. 4 days after γ-ray irradiation, proliferation of BMC was significantly decreased in LtriP-treated animals compared to the controls, where it was already weak (fig. 7). On day 14, normal proliferation was observed only with the BMC of LtriP-treated mice.

The proliferative response of SC to Con A was followed from day 1 to day 30 after irradiation. In controls, this response was abolished on day 1, reappeared around day 8 and was totally normal from day 18 on (data not shown). When animals were treated with LtriP, a significant difference was observed only from days 13 to 15, as shown in figure 8. When measured by [^{3}H] thymidine incorporation, a significant decrease was observed in the Con A response of SC from treated animals compared to that of controls. In contrast, the viable cell number, measured as MTT reduction, was found to have increased in spleens from LtriP-treated mice and Con A did not significantly modify the difference between LtriP-treated and control animals.

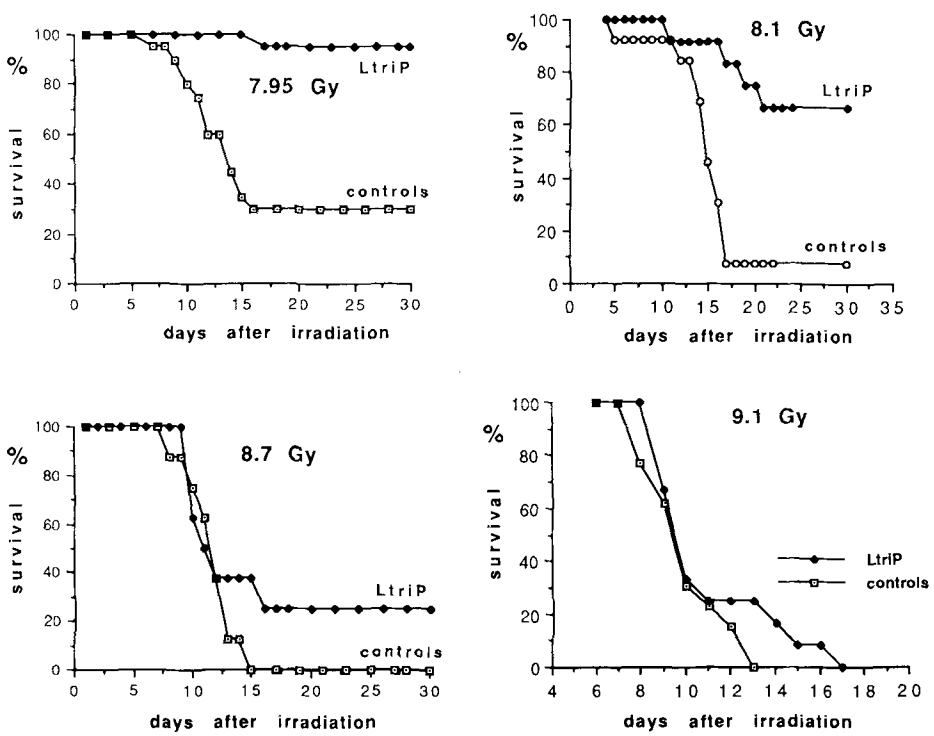

Figure 3. Effect of irradiation doses on the survival of NMRI mice treated i.p. with LtriP (0.5 mg/kg) or saline (controls) on 2 successive days before irradiation, n = 12.

Effect of LtriP (0.5 mg/kg i.p.) or 0.5 ml saline (control) on peripheral blood leukocyte counts of mice treated on 2 successive days before γ-ray irradiation (7.95 Gy); on survival (%, animal number = 50 per group)

Day after irradiation	Treatment	Leukocyte counts ($\times 10^2/mm^3$) Total	PMN	Lymphocytes	Monocytes	Mice survival at day 30 (%)
0	none	53.8 ± 8[a]	4.7 ± 1.2	46.6 ± 6.6	1.6 ± 0.5	100
1	control	8 ± 1.3	5.0 ± 1.5	2.9 ± 0.5	0.2 ± 0	100
	LtriP	10 ± 1.3	3.7 ± 0.8	**6.1 ± 0.8**[b]	0.3 ± 0.1	100
8	control	3 ± 0.4	0.3 ± 0.1	2.2 ± 0.4	0.1 ± 0	97.5
	LtriP	3 ± 0.4	0.5 ± 0.1	1.9 ± 0.1	0.1 ± 0	100
12	control	4 ± 2.2	0.3 ± 0	1.9 ± 0.4	0	75
	LtriP	3 ± 0	0.6 ± 0.1	2.2 ± 0.2	0	100
15	control	6 ± 2.7	0.2 ± 0	3.1 ± 0.4	0.1 ± 0	50
	LtriP	10 ± 2.2	**2.3 ± 0.6**[b]	7.8 ± 1.7	0.1 ± 0	100
18	control	41 ± 15	7.2 ± 4.6	32.5 ± 11.6	1.0 ± 0.6	35
	LtriP	47 ± 16	8.5 ± 3.2	36.6 ± 13.5	2.0 ± 1.3	95

[a]Each value represents the mean ± SE of 5 mice;
[b]Significantly different from control values (Student's t-test, $p < 0.05$).

Discussion

Data presented in this report demonstrate that the lauroyltripeptide LtriP conferred radioprotection on mice when injected before otherwise lethal γ-ray irradiation, as do other immunomodulators. This activity correlated with its capacity to stimulate the immune system, since the non-immunostimulating analog LtetraP D,DA_2pm, injected under the same conditions, was unable to protect irradiated mice. The radioprotection observed with LtriP was not due to contaminating LPS, as shown by the different response obtained with *S. enteritidis* LPS (fig. 2). Survival induced by LtriP was both time- and concentration-dependent. Optimum protection was obtained with 0.5 mg/kg (about 12 µg/animal) of LtriP injected on 2 successive days before irradiation.

Granulocytes reappeared earlier in LtriP-treated mice than in untreated mice, but the difference was significant only 15 days after irradiation. This time-point corresponded to the turning point for the fatal hemopoietic syndrome. Although no other significant difference was observed in other blood cells, histological analysis demonstrated that, starting at day 8, LtriP treatment accelerated recovery in bone marrow, spleen and thymus. These tissues had a nearly normal aspect at day 15, whereas much slower recovery was observed in control animals.

Four days post-irradiation, BMC from LtriP-treated mice were less responsive to serum containing M-CSF than were those from irradiated control mice. Fourteen

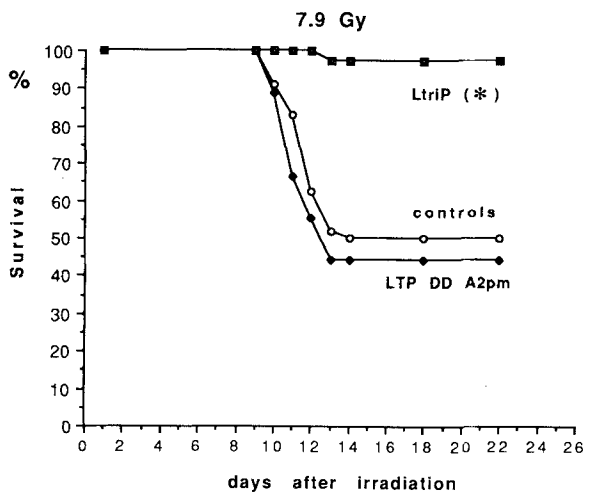

Figure 4. Survival of NMRI mice treated i.p. with LtriP and LtetraP DDA_2pm (0.5 mg/kg) or saline (controls) on 2 successive days before irradiation (7.9 Gy), n = 12.
*Significantly different from control value (Logrank test, $KHI_2 > 3.84$).

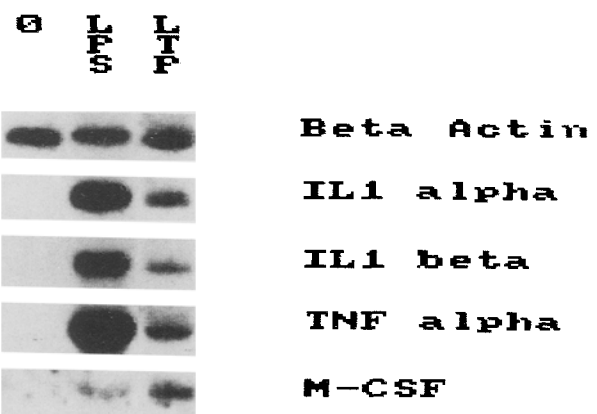

Figure 5. IL-1α, IL-1β, TNFα and M-CSF mRNA synthesis in peritoneal macrophages of $B_6D_2F_1$ mice after 6 h of LtriP (LTP) stimulation, or LPS stimulation (O = controls).

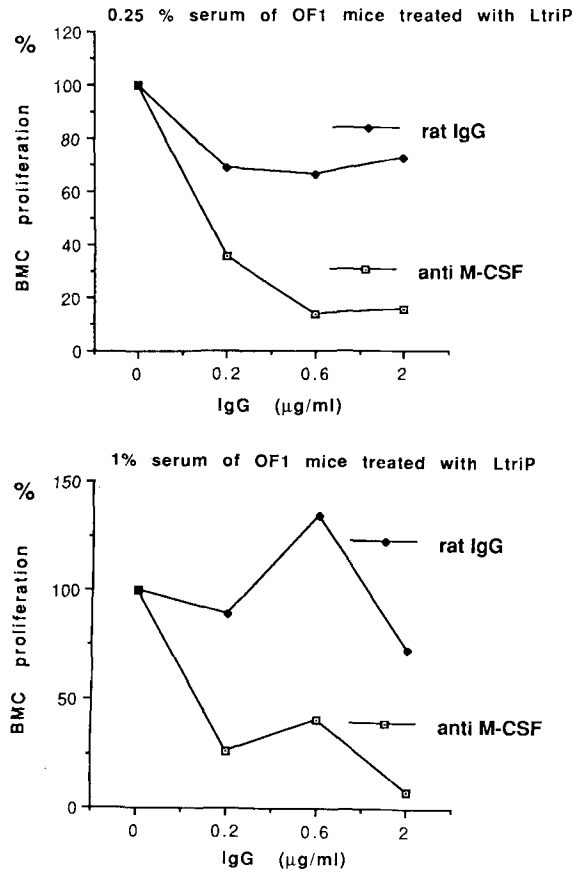

Figure 6. Proliferation of OF1 mouse BMC. Cells were cultured in 0.25% or 1% serum of OF1 mice treated with LtriP to which rat IgG or anti M-CSF antibodies at different concentrations were added. %OD controls with MTT determination.

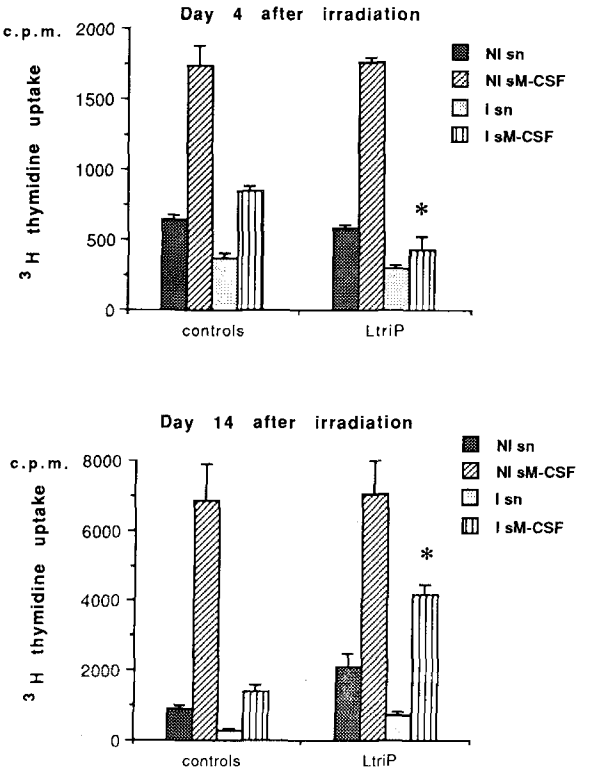

Figure 7. Proliferation of NMRI mouse BMC incubated in normal mouse serum (sn) or M-CSF mouse serum (sM-CSF) 4 days and 14 days after irradiation (7.95 Gy). Histograms represent the averaged results of 3 different experiments. I = irradiated mice; NI = non-irradiated mice.
*Significantly different from control values (Student's t-test, $p < 0.05$).

days post-irradiation, BMC from irradiated LtriP-treated mice, but not those from irradiated controls, had partially recovered the response to M-CSF, evaluated by comparison with that in non-irradiated cells.

Patchen et al. observed that in mice treated with *Saccharomyces cerevisiae* glucan, on days 3, 5 and 7 post-irradiation, the carbon-clearance capacity is reduced compared with that of controls, whereas host-resistance to microbial infection is enhanced[3]. 'Reticuloendothelial-system (RES) blockade' was previously described by Mori et al.[18] in their study of the radioprotective effect of carbon particles in mice. This RES blockade was reported to aid the recovery of hemopoiesis in irradiated mice by preventing the phagocytosis of slightly injured, yet still functional, hemopoietic stem cells[18]. Serum from carbon-treated mice contains not only CSF for macrophage populations but also factors(s) that inhibit granulopoiesis[19]. RES blockade results in the increased survival of pluripotent stem cells and permits hemopoietic cells to mature after the irradiation.

Similarly, on day 14 post-irradiation, we observed that even though the viability of SC of LtriP-treated mice was significantly higher than those of irradiated controls, their proliferative response to the T-cell mitogen Con A was weaker. This may reflect the appearance of a cell population unresponsive to Con A, such as precursors migrating from thymus or bone-marrow, or another lymphocyte population.

Recently, the expression of a deregulated bcl-2 gene in hemopoietic stem cells deprived of growth-factors was reported to prolong cell survival. Bcl-2, an inner mitochondrial protein, was demonstrated to block programmed cell death in selected hemopoietic cell lines following cytokine deprivation[21]. This protein is restricted to tissues characterized by apoptotic cell death, such as the thymus, where it is particularly expressed in the mature thymocytes of the medulla [22]. Overexpression of Bcl-2 was obtained in two different strains of transgenic mice. In vitro, thymocytes of the two transgenic mouse strains are protected from radiation[23,24] and in vivo, after γ-irradiation of transgenic mice, an enhanced survival of thymocyte populations is observed, particularly of cortical $CD_4^+CD_8^+$cells, which are rapidly eliminated in normal mice[24].

Carbon particles, glucan and LtriP are very different compounds which nevertheless act similarly to restore hemopoiesis after irradiation, probably by simulating

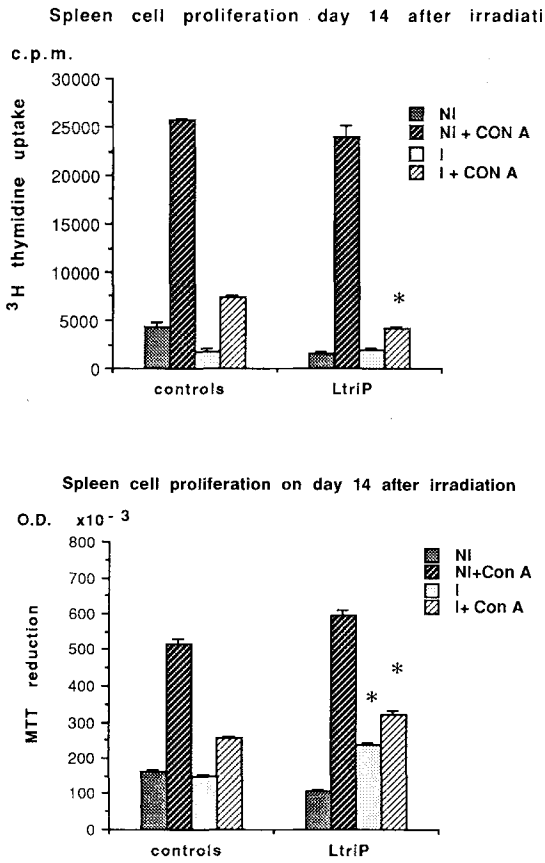

Figure 8. Proliferative spleen cell response of LtriP treated or untreated mice 14 days after irradiation (8.1 Gy), measured by ^{3}H thymidine incorporation and by MTT reduction. Histograms represent the average results of 6 different experiments. NI = non-irradiated mice; I = irradiated mice.
*Significantly different from control values (Student's t-test, $p < 0.02$).

immunological factors. Several cytokines, especially IL-1 and TNF-α, and growth factors have been reported to protect irradiated mice[5,19]. Recombinant human granulocyte-CSF enhanced the survival of mice irradiated with X-rays[20]. LtriP may exert its radioprotective effect by inducing these cytokines and M-CSF, as shown in this report. Immunostimulation by immunological factor(s) might induce upregulation of bcl-2, protecting cells from radiation injury. After a delay, maturation process might start again so that new hemopoietic cells would be formed.

Another mechanism of protection by LtriP may be involved. Previous studies[9] demonstrated that LtriP protects mice against the hepatotoxicity of paracetamol, which is due to cytochrome P-450-dependent formation of toxic radicals and metabolites. This protection could be related, at least to some extent, to a decrease in the amount of hepatic cytochrome P-450. The toxicity of ionizing radiation is mediated by free radical species, especially the oxygen-derived free radicals. These compounds, produced in large quantities in vivo, have been implicated as the cause of lipid peroxidation in liver microsomes[7]. IL-1 and other immunostimulants also influence levels of hepatic cytochrome P-450. The correlation between decreased lipid peroxidation and radioprotection may provide a new orientation for radioprotection studies.

Besides the interest of the immunomodulating lipopeptide LtriP as a possible adjuvant in radiotherapy, it may be a useful tool, because of its well defined molecular structure, in the studies of immunomodulation and radioprotection mechanisms that are now underway.

Abbreviations
A_2pm = diaminopimelic acid; DDA_2pm or LLA_2pm = D,D- or L,L-diaminopimelic acid; BCG = bacillus of Calmette and Guérin; BMC = bone marrow cells; Con A = concanavalin A; FCS = foetal calf serum; Gy = gray; IL-1 = interleukin 1; LPS = lipopolysaccharide (endotoxin); LtetraP = lauroyltetrapeptide; LtriP = lauroyltripeptide; M-CSF = macrophage-colony stimulating factor; MTT = (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue); RES = reticuloendothelial system; SC = spleen cells; TNF = tumor necrosis factor; WBC = white blood cells.

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