Timing in Administration of a Heat-killed Lactobacillus casei Preparation for Radioprotection in Mice

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A single subcutaneous injection of a preparation of heat-killed Lactobacillus casei (LC 9018), given before or after irradiation, significantly increased the survival rate of mice that had received 8.5-Gy ¹³⁷Cs whole-body γ-irradiation. A similar radioprotective effect was observed when LC 9018 was administered within the period from 2 days before irradiation to 9 h after irradiation, the pre-irradiation treatment being slightly better than the post-irradiation treatment. Increases in the weight of the spleen and in the number of endogenous spleen colonies on days 8 and 12 after irradiation suggested that the radioprotective effect was based on enhanced recovery of hematopoietic tissues. The activity of macrophage colony-stimulating factor (M-CSF) in serum was rapidly increased by the treatment and was maintained at the elevated level for 13 days. At the same time, an increased level of M-CSF mRNA was detected in the livers of the treated mice. However, LC 9018 failed to save the lives of mice when administered 3 days after irradiation, although it increased serum M-CSF as effectively as noted above. The small advantage of the pre-irradiation over the post-irradiation treatment was not explained by the increases of metallothionein in the hematopoietic tissues of the treated mice.

INTRODUCTION

A substantial amount of evidence now indicates the beneficial effects of recombinant hematopoietic growth factors, such as granulocyte colony-stimulating factor (G-CSF), granulocyte/macrophage colony-stimulating factor (GM-CSF), interleukin-3 and stem cell fac-

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tor, in accelerating the regeneration of hematopoietic tissues after experimental\textsuperscript{1–3}, clinical\textsuperscript{4,5}, or accidental\textsuperscript{6–8} exposure to ionizing radiation. However, these recombinant hematopoietic factors must be administered repeatedly for a fairly long period after irradiation, and moreover, the radioprotective effects of these factors were highly dependent on the schedule of administration both in the case of individual administration\textsuperscript{9,10} and in combined administration with another factor\textsuperscript{11}.

Interleukin-1 (IL-1), given as a single dose, also increases the survival rate of otherwise lethally irradiated mice, although it is much less radioprotective when administered 4 h before than when administered 20 h before irradiation\textsuperscript{12}. The effectiveness of many other radioprotective substances such as 16,16-dimethylprostaglandin E\textsubscript{2}\textsuperscript{13}, the immunomodulator AS101\textsuperscript{14}, the protein-associated polysaccharide AM5\textsuperscript{15}, and the bacterial extract Broncho-Vaxom\textsuperscript{R16}, also depends on the conditions of administration.

We have reported previously that a single subcutaneous dose of LC 9018 was highly radioprotective when given shortly after irradiation in mice\textsuperscript{17}. LC 9018 is a lyophilized preparation of heat-killed \textit{Lactobacillus casei} YIT 9018, a strain of nonpathogenic intestinal bacteria of human origin. In an earlier study than ours mentioned above, Aoki\textsuperscript{18} showed that a streptococcal preparation, OK-432, was radioprotective when given as a single intraperitoneal dose 2 days before or 10 h after irradiation. The OK-432 preparation contains penicillin to keep the bacteria in an inactive state.

In this present study, we investigated the radioprotective effects of LC 9018 when given before irradiation. Since LC 9018 does not contain any components other than actual bacterial cells, its effect must be entirely attributable to its effect as an immunomodulator.

\section*{MATERIALS AND METHODS}

\subsection*{Animals and irradiation}

Male C3H/HeV mice (10–11 weeks old) were used in all experiments. They were bred and maintained under specific pathogen-free conditions in the facilities of the National Institute of Radiological Sciences. They received whole body irradiation, the source being a $^{137}$Cs (Toshiba $\gamma$-ray Irradiator, Model RSG-50), at a dose rate of about 60 cGy/min. Other details are the same as those described previously\textsuperscript{17}.

\subsection*{Lactobacillus casei preparation (LC 9018)}

LC 9018 was prepared on a large scale by heating and lyophilizing strain YIT 9018 bacteria; it was stored in 10-mg portions. The preparation was suspended in saline shortly before use and injected subcutaneously into the inguinal region in mice.

\subsection*{Serum CSF assay}

Blood was collected by heart puncture after animals were anesthetized with ether. The sera of 4–6 mice in each group were pooled and stored at $-20^\circ$C. Before use in the assay, serum was heated at 56°C for 30 min to inactivate complements. Bone marrow cells were cultured by a
method described previously\(^17\). Briefly, bone marrow cells \((1 \times 10^5)\) were suspended in 1 ml of McCoy's 5A medium containing 20\% horse serum (GIBCO), 0.32\% agar (DIFCO), and 0.1 ml serum in a 35-mm culture dish (Lux) and they were then incubated at 37\(^\circ\)C under 5\% CO\(_2\) in humid air for 6–7 days. After incubation, colonies containing more than 50 cells were counted under an inverted microscope.

**Northern blot hybridization.**

Either one day or 8 days after irradiation, the mice were killed, and the livers, spleens, and bilateral femurs were removed; they were pooled on ice for each group of 5 mice. Liver and spleen were cut into small pieces with scissors and passed through a disposable mesh (Falcon 35–2350) in ice-cold saline. Bone marrow cells were suspended in ice-cold saline. The tissues and cells were washed by centrifugation and then frozen at \(-80^\circ\)C. Total RNA, extracted by a guanidine-hot phenol method, was subjected to Northern blot hybridization, as described previously\(^19\). Radioactive bands on the Northern blot were quantitatively analyzed with a BAS 2000 System using Imaging Plates\(^\text{TM}\) (Fuji Photo Film Co. Ltd., Kanagawa Japan) and corrected for the intensity of the band of \(\beta\)-actin in each blot. The following cDNA fragments were used as probes: a 239-base pair fragment of human M-CSF cDNA; a 208-base pair fragment of murine GM-CSF cDNA; a 500-base pair fragment of murine G-CSF cDNA, generously provided by Dr. S. Nagata (Osaka Bioscience Industries, Osaka); and a 300-base pair fragment of the human \(\beta\)-actin gene (Wako Pure Chemicals, Tokyo). Using the reverse transcriptase-PCR method based on the nucleotide sequence databases, we cloned the above-mentioned human M-CSF cDNA and murine GM-CSF cDNA probe from the RNA of the U-937 human cell line and of murine bone marrow, respectively.

**Endogenous spleen colony**

Eight or 12 days after irradiation (8.5 Gy), the mice were sacrificed and the spleens were removed, weighed, and fixed in Bouin's solution. The number of colonies visible on the spleen surface was counted.

**Determination of metallothionein**

The method of Kotsonis\(^20\) was used. Briefly, spleens, livers and bilateral femurs were removed from the treated mice. The spleen or a portion (200 mg) of liver was homogenated in 2 ml of 1.15 M KCl in Teflon-glass homogenizers. Bone marrow cells were suspended in 2 ml of 0.1 M Tris-HCl (pH 7.6) and disrupted with a sonicator (TAITEC VP-5) for 15 sec. The homogenated or sonicated tissues were pretreated with dimethylmalate and CdCl\(_2\), and then heated at 100\(^\circ\)C for 5 min. After the addition of 50 nmoles (spleen or liver) or 5 nmoles (bone marrow) of \(^{203}\)Hg(NO\(_3\))\(_2\) (NEN Research Products; specific activity, 51.4 MBq/mg), ovalbumin and TCA were added to remove excess \(^{203}\)Hg ions. Radioactivity was determined with an autogamma counter (ARC-500). Metallothionein content was computed from the remaining radioactivity in the supernatant, based on the assumption that one mole of metallothionein binds 7 moles of mercury ions. The protein content of the bone marrow cell preparation was determined with the Bio-Rad Protein Assay, using BSA as a standard.
Other methods

The effects of LC 9018 on spleen weight, spleen colony number and metallothionein content were analyzed by Student’s t-test. For the analysis of the survival rate of irradiated mice, we used the generalized Wilcoxon test. Antisera for mGM-CSF and mM-CSF were generously provided by Dr. E. R. Stanly (Albert Einstein College of Medicine, New York) and Dr. T. Sudo (Tohre Co. Ltd., Kanagawa Japan), respectively. A separate experiment showed that 0.33 μl of anti-M-CSF antiserum and 0.08 μl of anti-GM-CSF antiserum respectively were sufficient to completely inhibit 100 units of standard mM-CSF (L-cell CSF)21 and rmGM-CSF (a gift from Sumitomo Pharmaceutical Co., Osaka) in the above-mentioned bone marrow cell culture.

RESULTS

Timing of LC 9018 Administration and Survival Rate of Irradiated Mice

In Group-I mice, which received a single subcutaneous LC 9018 injection 6, 9, 15, 24, 32 or 48 h before 8.5-Gy total-body irradiation, an average of 96% survived for 30 days after irradiation, whereas 93% of the mice in the control group which received the saline injection died within 20 days (Fig. 1). Compared with Group-I mice, an average of 89% of the mice in Group-II, which received a LC 9018 injection 0.1, 3, 6, or 9 h after irradiation, survived. The difference between Group-I and Group-II was significant (P<0.05), indicating that LC 9018 was somewhat more effective when used before than after irradiation. The survival rate in a group of mice that received LC 9018 3 days before irradiation was 60%. Although this rate was

![Fig. 1. Effects of timing of LC 9018 administration on the mortality of irradiated mice. LC 9018 (1 mg in 0.1 ml saline) was injected in a single subcutaneous dose on various days before (−) or after (+) irradiation, as indicated. The 30-day survival rate was 7% for the 100 control animals that received saline immediately after 8.5-Gy irradiation. Each point represents values for 20–100 mice.](image-url)
significantly larger than that in the control saline-treated group (p<0.01), the life-saving effect of LC 9018 in this group was less marked than its effect in Group-I (p<0.01). The effect of LC 9018 was further reduced when it was given 7 days before or 30 h after irradiation (Fig. 1). These findings indicate that to achieve radioprotection with LC 9018, the timing of administration before or after irradiation is limited. Nevertheless, the increased survival rate in 7-day pre-irradiation or 30-h post-irradiation treated mice was still significantly better (p<0.01 or 0.05, respectively) than the survival rate (7%) in the saline-treated mice.

**Serum Colony-Stimulating Factor Activity after Treatment of Irradiated Mice with LC 9018**

The injection of LC 9018 in irradiated mice resulted in a long-term elevation of serum CSF activity (Fig. 2). The CSF activity increased significantly within several h after the injection of LC 9018, and remained elevated for up to 13 days thereafter in all three groups of mice that received LC 9018 immediately (panel b), 1 day (panel c), or 3 days (panel d) after irradiation. The increase in serum CSF was greater when the administration of LC 9018 was delayed. Similar long-term elevation of serum CSF was observed in those mice that had received LC 9018 24 h before irradiation (data not shown). In all the serum preparations mentioned above, more than 90% of the CSF activity was inhibited by the anti-M-CSF antiserum, while less than 10% was
inhibited by the anti-GM-CSF antiserum (data not shown). By contrast, CSF activity was undetectable in the serum of saline-treated mice within 8 days after irradiation (panel a).

Northern blot analysis revealed that M-CSF mRNA was increased in the livers of mice that had received LC 9018 immediately after irradiation (Fig. 3). The increase was detected 24 h after the treatment and continued for up to 8 days after the treatment. Intensity of M-CSF mRNA relative to that of β-actin mRNA was 0.1 in the treated mice, whereas M-CSF mRNA was barely detectable in the saline-treated mice. A similar increase in M-CSF gene expression was observed in the livers of mice that had received LC 9018 at various points of time after irradiation (data not shown). GM-CSF mRNA on the other hand was detectable only in spleen on Day 8 after irradiation in the LC 9018 treated-mice (relative intensity, 0.09). The observation of an early increase in M-CSF mRNA was consistent with the early increase in serum M-CSF activity in the treated animals. G-CSF mRNA was detectable neither on Day 1 nor on Day 8 after irradiation. In bone marrow cells, mRNA for these CSF was not detected in any preparations examined (data not shown).

**Fig. 3.** Expression of the CSF gene in tissues of irradiated mice that received LC 9018 (1 mg) or saline immediately after 8.5-Gy irradiation. Tissues were collected 1 or 8 days after irradiation and were pooled for each group of 5 mice. Each lane, 10 μg total RNA. Molecular size of CSF mRNA was estimated by the mobility of 0.24–9.5 Kb RNA Ladder (BRL).
Enhancement of Hematopoietic Recovery in Irradiated Mice

The effects of LC 9018 on hematopoiesis when given pre- and post-irradiation were compared. LC 9018 enhanced the recovery of both spleen weight and endogenous spleen colonies, regardless of whether it was administered 24 h before or immediately after 8.5-Gy irradiation (Table 1). Findings obtained 12 days after irradiation were consistent with those obtained 8 days after irradiation. A difference between the pre- and post-irradiation treatments was observed only with regard to the number of Day-8 spleen colonies (p<0.05).

Table 1. Effects of LC 9018 in Spleen Weight and on the Number of Endogenous Spleen Colonies in Irradiated Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 8</th>
<th>Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spleen (mg)</td>
<td>Colonies (per spleen)</td>
</tr>
<tr>
<td>Saline at 0.1 h</td>
<td>26.7±3.8</td>
<td>0.1±0.3</td>
</tr>
<tr>
<td>LC at 0.1 h</td>
<td>31.5±5.3*</td>
<td>1.3±1.8</td>
</tr>
<tr>
<td>LC at −24 h</td>
<td>34.6±4.9**</td>
<td>3.1±1.8***</td>
</tr>
</tbody>
</table>

| Saline (0.1 ml) or LC 9018 (1 mg in 0.1 ml saline) was injected subcutaneously 0.1 h after or 24 h before 8.5-Gy whole body irradiation. |
| Mean±SD for 10 mice. |
| Asterisks indicate significant differences from saline treatment; p<0.05 (*), p<0.01 (**), p<0.001 (***). |

Table 2. Induction of Metallothionein in Mice after Injection of LC 9018 or Cadmium Chloride

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver (per g tissue)</th>
<th>Spleen (per g tissue)</th>
<th>Bone marrow (per g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>10.5±1.4*</td>
<td>15.9±1.4</td>
<td>21.8±14.4</td>
</tr>
<tr>
<td>LC 9018</td>
<td>162±45*</td>
<td>13.9±2.0</td>
<td>23.5±5.1</td>
</tr>
<tr>
<td>CdCl₂</td>
<td>435±26*</td>
<td>49.1±14.4*</td>
<td>87.9±3.9*</td>
</tr>
</tbody>
</table>

| LC 9018 (1 mg) or cadmium chloride (150 μg) was injected subcutaneously in 0.1 ml saline 24 h before whole body irradiation (8.5 Gy). Control animals received 0.1 ml saline alone. |
| Mean±SD for 5 mice. |
| Significant difference from saline treatment (p<0.001). |

Tissue Metallothionein Content after Treatment with LC 9018

LC 9018 significantly increased the metallothionein content in liver (Table 2). However, there was no increase in the metallothionein content in the spleen and bone marrow of LC-9018 treated mice. By contrast, the effect of cadmium chloride was observed not only in liver but also in spleen and bone marrow.
DISCUSSION

This study showed that a single dose of LC 9018 was radioprotective, even when administered as late as 30 h after or as early as 7 days before irradiation. The extent of the radioprotective effect was similar when LC 9018 was administered during the period from 2 days before irradiation to 9 h after irradiation, although the pre-irradiation treatment was slightly more effective than the post-irradiation treatment. The latitude of the effective timing of administration was 4 times greater for the pre-irradiation treatment than for the post-irradiation treatment.

Compared with the transient increase in serum CSF activity that was induced either by LC 9018 alone or by irradiation alone, the administration of LC 9018 in the irradiated mice resulted in the long-term elevation of serum CSF. This effect appears to aid the enhancement of the recovery of hematopoietic tissues in the irradiated mice. It seems that the continuous elevation of serum CSF activity begun within 1 or 2 days after irradiation was necessary for saving the lives of the irradiated mice, because LC 9018 failed to save the lives of the irradiated mice when administered 3 days after irradiation, although it was sufficiently effective in increasing serum CSF. LC 9018 increases hematopoietic precursor cells in spleen and bone marrow as we reported previously. The present observation indicates that such hematopoietic recovery must be started within a few days after irradiation. Delayed increase in CSF is helpless in this regard.

Because the pre-irradiation treatment with LC 9018 was more effective than the post-irradiation treatment (Fig. 1, Table 1), we speculated that the pre-irradiation treatment may have increased amounts of the radical scavenger, metallothionein, in the hematopoietic tissues. In a previous study, Matsubara et al. indicated that the induction of metallothionein in the tissues of mice pre-treated with cadmium or manganese salts was an important factor in the mechanism where by these metallic compounds provided radioprotection. Our present study showed that LC 9018 mimicked CdCl₂ in increasing metallothionein concentration in the liver, but that it differed from CdCl₂ in that it did not cause similar metallothionein increases in hematopoietic tissues. The metallothionein induced in the LC 9018-treated mice may play a part in the preservation of essential metalloelements, facilitating the de novo synthesis of metalloelement-dependent enzymes rather than acting as a radical scavenger.

The slightly better protection offered by the pre-irradiation treatment could be explained in terms of changes in the cell cycle, or it could be due to some other, unknown, reasons.

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REFERENCES


