Ionizing Radiation and Bacterial Challenge Alter Splenic Cytokine Gene Expression

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Irradiation increases susceptibility to bacterial infection. Exogenous proinflammatory cytokines can alter the response of mice to γ radiation, but the role of endogenous inflammatory cytokines after bacterial infection in irradiated animals is not known. Gene expression of hematopoietic (GM-CSF) and proinflammatory (IL-1β, IL-6 and TNF-α) cytokines were examined in spleens of B6D2F1/J female mice after irradiation alone (1.0- and 7.0-Gy), and after irradiation followed by Klebsiella pneumoniae s.c. challenge 4 days postirradiation by using the reverse transcription-polymerase chain reaction (RT-PCR) and Southern blot hybridization. At 4, 8, and 24 h after bacterial challenge in 7.0-Gy-irradiated mice, GM-CSF mRNA increased (p < 0.05). TNF-α mRNA in irradiated mice were slightly decreased, whereas after bacterial challenge, TNF-α mRNA elevated at 30 h in 7.0-Gy-irradiated mice; at 4, and 8 h in 1.0-Gy-irradiated mice, and at 1 h in sham-irradiated mice (p < 0.05). IL-6 mRNA displayed a biphasic response in 7.0-Gy-irradiated mice, and, after bacterial challenge, in both irradiated mice (1.0- and 7.0-Gy) and sham-irradiated mice. IL-1β mRNA remained at or below normal for 8 h and increased at 24 h after bacterial challenge on day 4 in 7.0-Gy-irradiated mice. These results indicate that sublethal gamma radiation alters the patterns of the hematopoietic and proinflammatory cytokine responses to bacterial challenge in vivo. Consequently, treatment protocols may need to take into account changes in cytokine gene responses to resolve infection after irradiation.

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Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-1β, interleukin-1β; IL-6, interleukin-6; TNF-, tumor necrosis factor- ; Taq, Thermus aquaticus; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger ribonucleic acid; cDNA, complementary deoxyribonucleic acid
INTRODUCTION

It is well established that infection is a major cause of death after whole-body ionizing radiation injury\(^1\) and that endogenous and exogenous cytokines play a major role in controlling the host’s responses to radiation and infection\(^2\). Some cytokines are produced constitutively, but most are produced by induction\(^3\). Cytokines are produced by several different cell types, including monocytes/macrophages, endothelial cells, T-lymphocytes, fibroblasts, and polymorphonuclear leukocytes\(^4\). The first line of defense against bacterial infection involves the initiation of an inflammatory response and the release of mediators such as oxygen radicals and proinflammatory cytokines from activated macrophages and granulocytes\(^5\). The role of endogenous cytokines in response to radiation and bacterial infection is not clear, but administration of neutralizing antibodies against cytokines in irradiated mice has indicated that interleukin (IL)-1 and tumor necrosis factor (TNF) in blood and tissue contribute to radioresistance in mice\(^6\). Further, several investigators demonstrated that endogenous production of IL-6 and TNF-\(\alpha\) is a critical component of antibacterial responses\(^7,8\). Also, major trauma and sepsis may lead to overproduction of TNF, IL-1, and IL-6, which may have deleterious actions on metabolic functions and hemodynamic stability\(^9\). Neutralizing antibodies to IL-6 and TNF-\(\alpha\) attenuate deleterious consequences of Gram-negative sepsis caused by bacterial endotoxin\(^10\). Thus, an understanding of the patterns of cytokine production in a number of experimental and clinical situations could contribute to development of improved treatment protocols. Monitoring of cytokines could correlate with efficacy of therapy.

The purpose of this study was to investigate the change in expression of a single hematopoietic cytokine gene, granulocyte-macrophage colony-stimulating factor (GM-CSF), and three proinflammatory cytokine genes (IL-1\(\beta\), TNF-\(\alpha\), and IL-6) in mouse spleen after irradiation and during bacterial challenge after sublethal gamma radiation. The spleen is a hematopoietic organ in mice. The hypothesis tested was that the gene expression of hematopoietic and proinflammatory cytokines in response to an infectious challenge is both qualitatively and quantitatively different in irradiated and sham-irradiated mice. Little is known about cytokine gene expression in vital hematopoietic organs after irradiation and subsequent bacterial challenge. In this study we used the reverse transcription-polymerase chain reaction (RT-PCR) and Southern blot hybridization techniques to determine cytokine mRNA both in mice after irradiation alone and in mice that were irradiated and then challenged with \(K.\ pneumoniae\).

MATERIALS AND METHODS

Mice and irradiation conditions

All animal experiments were approved by the institutional animal care and use committee. Research was conducted according to the principles enunciated in the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources,
National Research Council (11). B6D2F1/J female mice were purchased from Jackson Laboratories (Bar Harbor, ME) and weighed approximately 20 g when irradiated in our 60Co gamma radiation facility. Irradiation was performed bilaterally delivering total absorbed doses of 1.0 Gy and 7.0 Gy at a dose-rate of 0.4 Gy/min. In this strain of mouse, 1.0 Gy is a low sublethal dose, while 7.0 Gy is a high sublethal dose (12). Sham-irradiated mice were treated similarly to irradiated mice but were not irradiated. Dosimetry was performed as described previously (12,13).

**Bacterial challenge**

*K. pneumoniae* serotype 5 AFRRI 7 was obtained from a clinical isolate and prepared as previously described (14). Four days after irradiation, mice were lightly anesthetized with methoxyflurane, and challenged s.c. with a number of *K. pneumoniae* that was an approximate LD₈₅/₉₀ for mice at each dose of gamma radiation, i.e., 7.0-Gy-irradiated mice were given 1.8 x 10⁵ colony forming units (CFU)/0.2 ml; 1.0-Gy-irradiated mice, 4.2 x 10⁶ CFU/0.2 ml; and sham-irradiated mice, 7.5 x 10⁶ CFU/0.2 ml. Four days after irradiation, at the time of challenge with bacteria, peripheral blood and bone marrow cellular elements are maximally depressed (12). The challenge doses of bacteria used assured an equivalent survival response of mice regardless of the dose of radiation.

**LD₅₀/₃₀ *K. pneumoniae* in irradiated mice**

Five sets of 16 mice each were either sham-irradiated (0 Gy) or given 1.0, or 7.0 Gy radiation. Four days after irradiation, each set of 16 mice was challenged s.c. with an appropriate ten-fold serial dilution of *K. pneumoniae*. Survival was recorded for 30 days after bacterial challenge. A probit analysis of mortality provided the lethal dose of *K. pneumoniae* for 50% of mice (LD₅₀/₃₀).

**Experimental design and Statistics**

Initial studies were carried out in which each specific cytokine mRNA was evaluated in the spleens of mice each 1, 4, 7, 10, 14, and 17 days after 1.0-Gy- and 7.0-Gy-irradiation, respectively. In addition in this series of studies, two sham-irradiated mice, referred to as sham I controls, were evaluated at each time point. The data from sham-irradiated mice (n = 6 - 12) were pooled and are represented in the figures as a straight line. Subsequent studies were performed in which each specific cytokine mRNA was evaluated in 5 spleens each from 1.0-Gy- and 7.0-Gy-irradiated mice and sham-irradiated mice at 1, 4, 8, 24, 30, and 48 h after bacterial challenge, respectively. In this series of studies, sham-irradiated, non infected mice are referred to as sham II controls. As in the initial studies, two sham-irradiated mice were evaluated at all sampling times and data from the sham-irradiated mice were pooled. Comparisons with sham I controls at each time postirradiation and with sham II controls were made using Student’s t-test. In the second series of experiments comparisons were also made between irradiated-infected mice and sham-irradiated-infected mice. Transformations of the data, where needed, were made to achieve homogeneous variance. The y-axis scales in the figures reflect these transformations. Statistical analyses were done using Systat 7 (SPSS Inc., Chicago, IL). The specificity of cytokine mRNA was determined by the ratio of mRNA for each
cytokine to mRNA for the housekeeping gene GAPDH for each animal. The ratio of cytokine mRNA to GAPDH mRNA in normal controls did not vary significantly (p > 0.05) between experiments. Therefore, this ratio was used as the reference point within each experiment.

**RNA preparation and RT-PCR**

Whole spleens were homogenized in 2 ml of RNA STAT-60 (Tel-Test Inc., Friendswood, TX) with a polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY). Homogenized spleens were immediately frozen in liquid nitrogen and kept in a −70°C freezer until mRNA analysis. Total RNA was extracted according to the manufacturer’s protocol as modified by Chomczynski and Sacchi\(^{15}\). RNA concentrations were determined spectrophotometrically at 260 nm (Beckman DU-65). Total RNA for each sample was characterized by ethidium bromide-staining of agarose gels to ensure use of intact total RNA only. Intact total RNA was reverse transcribed into complementary deoxyribonucleic acid (cDNA). The final volume of 2.5 µl reverse transcription reaction mixture contained 0.5 µg total RNA, 0.5 µl reverse transcription reaction buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl and 3 mM MgCl\(_2\), 0.2 µl of 100 mM DTT, 0.05 µl of 40 U/ml RNAsin (Promega, Madison, WI), 0.2 µl of 25 U/µl random primer PD(N)\(_6\) (Boehringer Mannheim, Indianapolis, IN), and 0.12 µl of 200 U/µl Moloney murine leukemia virus reverse transcriptase (Life Technologies, Grand Island, NY). The reaction was carried out at 37°C for 1 h, stopped at 90°C for 10 min and then chilled on ice for 10 min. The synthesized cDNA was used as a template for the PCR. Primers for GM-CSF, IL-1β, IL-6 and TNF-α were purchased from Clontech Laboratories, Inc., Palo Alto, CA, and GAPDH was purchased from Synthetic Genetics, San Diego, CA. The sequence of primers and expected sizes of each cytokine are as follows:

- **GM-CSF (368 bp)**
  - 5’-primer: 5’TGTGGTCTACAGCCCTCTCAAGC3’
  - 3’-primer: 5’CAAGGGGATATCAGTCAAAAGGT3’

- **IL-1β (563 bp)**
  - 5’-primer: 5’ATGGCAACTGTTCCTGAACTCAACT3’
  - 3’-primer: 5’CAGGACAGGTATAGATTCTTCTTTT3’

- **IL-6 (638 bp)**
  - 5’-primer: 5’ATGAAGTTCCTCTCTGCAAGAGACT3’
  - 3’-primer: 5’CAGGACAGGTATAGATTCTTCTTTT3’

- **TNF-α (354 bp)**
  - 5’-primer: 5’TTCCTGTCTAAGCTGGTGGGTAGCTCGGCTC3’
  - 3’-primer: 5’GTATGAGATAGCCTACGCTCTGCGG3’

The reaction mixture for PCR contained 5 µl cDNA template from the RT reaction, 10 mM Tris-HCl (pH 8.3), 50mM KCl, 1.5 mM MgCl\(_2\), 0.8 mM each of dATP, dCTP, dGTP, and dTTP, 1.0 µM 5’-primer, 1.0 µM 3’-primer, and 1.25 U Taq (Thermus aquaticus) DNA polymerase. PCR was performed with a DNA Thermal Cycler (Perkin Elmer-Cetus, Norwalk, CT). PCR reactions were carried out on cDNA templates prepared in the absence of reverse transcriptase as negative controls. A positive control for each cytokine was also included in all PCR reactions. Cycle number was optimized individually for each cytokine as described by Gause and Adamovicz\(^{16}\). The amplified products were characterized in 1% agarose gel electrophoresis, transferred onto a Nytran nylon membrane (Schleicher and Schull, Keene, NY), and hybridized with the respective fluorescein-labeled GM-CSF, IL-6, TNF-α, GAPDH plasmid cDNA probe, and IL-1β oligonucleotide probe. The probes for IL-6, TNF-α, and GAPDH...
were prepared as described previously \(^{17}\). The GM-CSF probe consisted of a 0.8-kb insert cloned into the EcoRI site of pXM (Genetics Institute, Cambridge, MA). Probes for GM-CSF, IL-6, TNF-\(\alpha\) and GAPDH were labeled with a random priming commercial kit (Amersham, Arlington Heights, IL). The probe for IL-1\(\beta\) was labeled using an ECL 3'-oligolabelling commercial kit (Amersham, Arlington Heights, IL). Hybridization, stringency washes, and signal detection were carried out according to the manufacturer’s instructions. Fluorographs were prepared at room temperature on Kodak XAR film. The densitometric volume of the PCR product band of each sample was measured using a scanning laser densitometer (Molecular

![Graph](image)

**Fig. 1A.** Relative splenic GM-CSF mRNA determined by RT-PCR and Southern blot hybridization from mice 1, 4, 7, 10, 14 and 17 days after 1.0-Gy- and 7.0-Gy-irradiation. Data are expressed as the ratio of GM-CSF to GAPDH and represent the mean \(\pm\) SEM from three to five mice per treatment group and twelve mice for sham I controls group. Individual data points are not significantly different. The time period between vertical dashed lines is equivalent to that in Fig. 4A.

![Fluorograph](image)

**Fig. 1B.** Representative fluorograph of RT-PCR-amplified mRNA transcripts of GM-CSF and GAPDH in the spleens of sham I controls and 7.0-Gy-irradiated mice at days 1, 4 and 10. Each lane indicates mRNA transcript from an individual mouse.
Dynamics, Sunnyvale, CA). Semiquantitation was based on relative cytokine mRNA differences between treatment and control samples which were normalized to its GAPDH value.

RESULTS

Relative GM-CSF, IL-6, and TNF-α mRNA in spleens after irradiation

Relative GM-CSF mRNA responses increased ($p > 0.05$) up to 10 days after 7.0 Gy (Fig. 1A) when compared to sham I controls. No significant changes in GM-CSF mRNA was observed

![Graph](https://example.com/graph.png)

**Fig. 2A.** Relative splenic IL-6 mRNA determined by RT-PCR and Southern blot hybridization from mice 1, 4, 7, 10, 14 and 17 days after 1.0-Gy- and 7.0-Gy-irradiation. Data are expressed as the ratio of IL-6 to GAPDH and represent the mean ± SEM from three to five mice per treatment group and twelve mice for sham I controls group. *$p < 0.05$ and **$p < 0.01$ with respect to the sham I control value. The time period between vertical dashed lines is equivalent to that in Fig. 6A.

![Image](https://example.com/image.png)

**Fig. 2B.** Representative fluorograph of RT-PCR-amplified mRNA transcripts of IL-6 and GAPDH in the spleens of sham I controls and 7.0-Gy-irradiated mice at days 1, 4 and 10. Each lane indicates mRNA transcript from an individual mouse.
in 1.0-Gy-irradiated mice. A representative fluorograph of RT-PCR-amplified mRNA transcripts of GM-CSF and GAPDH in spleens of sham I controls and 7.0-Gy-irradiated mice at days 1, 4, and 10 is shown in Fig. 1B.

Relative IL-6 mRNA responses were significantly increased (p < 0.05) above sham I control values (n = 12) 1, 4, and 10 days only after 7.0 Gy. The increases in IL-6 mRNA occurred in a biphasic pattern, with peaks at days 1 and 10 (Fig. 2A). A representative fluorograph of RT-PCR-amplified mRNA transcripts of IL-6 and GAPDH in spleens of sham I controls and 7.0-Gy-irradiated mice at days 1, 4 and 10 is shown in Fig. 2B.

![Fig. 3A](https://example.com/fig3a.png)

**Fig. 3A.** Relative splenic TNF-α mRNA determined by RT-PCR and Southern blot hybridization from 1, 4, 7, 10, 14 and 17 days after 1.0-Gy- and 7.0-Gy-irradiation. Data are expressed as the ratio of TNF-α to GAPDH and represent the mean ± SEM from three to five mice per treatment group and nine mice for sham I controls group. Individual data points are not significantly different. The time period between vertical dashed lines is equivalent to that in Fig. 7A.

![Fig. 3B](https://example.com/fig3b.png)

**Fig. 3B.** Representative fluorograph of RT-PCR-amplified mRNA transcripts of TNF-α and GAPDH in the spleens of sham I controls and 7.0-Gy-irradiated mice at days 1, 4 and 10. Each lane indicates mRNA transcript from an individual mouse.
Relative TNF-α mRNA of irradiated mice were not different from sham I control values on the days tested (Fig. 3A). A representative fluorograph of RT-PCR-amplified mRNA transcripts of TNF-α and GAPDH in spleens of sham I controls and 7.0-Gy-irradiated mice at days 1, 4, and 10 is shown in Fig. 3B. The change in relative IL-1β mRNA was not determined in this experimental set. In Figs. 1A, 2A, 3A the time period of days 4 to 6 after radiation is indicated by dashed vertical lines and is equivalent to the 0–48 h time period after

Fig. 4A. Relative splenic GM-CSF mRNA determined by RT-PCR and Southern blot hybridization from sham-irradiated mice and 1.0-Gy- and 7.0-Gy-irradiated mice 1, 4, 8, 24, 30, and 48 h after K. pneumoniae challenge. Data are expressed as the ratio of GM-CSF to GAPDH and represent the mean ± SEM from three to five mice per treatment group and six mice for sham II controls group. *p < 0.05 and **p < 0.01 with respect to the sham II control value.

Fig. 4B. Representative fluorograph of RT-PCR-amplified mRNA transcripts of GM-CSF and GAPDH in the spleens of sham II controls and 7.0-Gy-irradiated-infected mice at 1, 4, 8 and 24 h. Each lane indicates mRNA transcript from an individual mouse.
bacterial challenge noted in Figs. 4A, 6A and 7A, respectively.

Relative GM-CSF, IL-1β, IL-6, and TNF-α mRNA in spleens after irradiation and K. pneumoniae challenge

Relative GM-CSF mRNA in spleens of 7.0-Gy-irradiated and bacteria-challenged mice was significantly elevated 2.6-, 3.2- and 2.1- fold (p < 0.05) above sham II controls (n = 6) 4, 8 and 24 h after bacterial challenge. The responses of 1.0-Gy-irradiated-infected mice were

![Graph showing relative GM-CSF mRNA levels over time](https://example.com/graph1.png)

**Fig. 5A.** Relative splenic IL-1β mRNA determined by RT-PCR and Southern blot hybridization from sham-irradiated mice and 1.0-Gy- and 7.0-Gy-irradiated mice 1, 4, 8, 24, 30, and 48 h after K. pneumoniae challenge. Data are expressed as the ratio of IL-1β to GAPDH and represent the mean ± SEM from three to five mice per treatment group and six mice for sham II controls group. *p < 0.05 with respect to the sham II control value.

![Fluorograph showing RT-PCR amplification](https://example.com/fluorograph.png)

**Fig. 5B.** Representative fluorograph of RT-PCR-amplified mRNA transcripts of IL-1β and GAPDH in the spleens of sham II controls, sham-irradiated-infected mice at 4 h, and 7.0-Gy-irradiated-infected mice at 24, 30 and 48 h. Each lane indicates mRNA transcript from an individual mouse.
similar to those of sham-irradiated-infected mice, except that GM-CSF mRNA in sham-irradiated-infected mice increased ($p < 0.05$) at 1 hr over sham II controls ($n = 6$) (Fig. 4A). A representative fluorograph of RT-PCR-amplified mRNA transcripts of GM-CSF and GAPDH in

![Graph showing relative splenic IL-6 mRNA determined by RT-PCR and Southern blot hybridization from sham-irradiated mice and 1.0-Gy- and 7.0-Gy-irradiated mice 1, 4, 8, 24, 30, and 48 h after $K$. pneumoniae challenge. Data are expressed as the ratio of IL-6 to GAPDH and represent the mean ± SEM from three to five mice per treatment group and six mice for sham II controls group. *$p < 0.05$ and **$p < 0.01$ with respect to the sham II control value.](https://academic.oup.com/jrr/article-abstract/41/3/259/1013713)

**Fig. 6A.** Relative splenic IL-6 mRNA determined by RT-PCR and Southern blot hybridization from sham-irradiated mice and 1.0-Gy- and 7.0-Gy-irradiated mice 1, 4, 8, 24, 30, and 48 h after $K$. pneumoniae challenge. Data are expressed as the ratio of IL-6 to GAPDH and represent the mean ± SEM from three to five mice per treatment group and six mice for sham II controls group. *$p < 0.05$ and **$p < 0.01$ with respect to the sham II control value.

**Fig. 6B.** Representative fluorograph of RT-PCR-amplified mRNA transcripts of IL-6 and GAPDH in the spleens of sham II control, sham-irradiated-infected mice at 4 h, 1.0-Gy-irradiated-infected mice at 4 and 48 h, and 7.0-Gy-irradiated-infected mice at 24 h and 30 h. Each lane indicates mRNA transcript from an individual mouse.
spleens of sham II controls and 7.0-Gy-irradiated-infected mice at 1, 4, 8 and 24 h is shown in Fig. 4B.

Relative IL-1β mRNA in 1.0-Gy- and sham-irradiated mice decreased below sham II controls at 1 h after bacterial challenge, then increased to a higher level at 4 h and then decreased again. Relative IL-1β mRNA in 7.0-Gy-irradiated-infected mice were different from those in 1.0-Gy- and sham-irradiated-infected mice. Relative IL-1β mRNA in the 1.0-Gy- and

![Graph showing the relative splenic TNF-α mRNA over time for different treatments](https://example.com/graph.png)

**Fig. 7A.** Relative splenic TNF-α mRNA determined by RT-PCR and Southern blot hybridization from sham-irradiated mice and 1.0-Gy- and 7.0-Gy-irradiated mice 1, 4, 8, 24, 30, and 48 h after K. pneumoniae challenge. Data are expressed as the ratio of TNF-α to GAPDH and represent the mean ± SEM from three to five mice per treatment group and six mice for sham II controls group. *p < 0.05 and **p < 0.01 with respect to sham II control value.

![Fluorographs of RT-PCR amplified mRNA transcripts](https://example.com/fluorograph.png)

**Fig. 7B.** Representative fluorograph of RT-PCR-amplified mRNA transcripts of TNF-α and GAPDH in the spleens of sham II controls, sham-irradiated-infected mice at 1 h, 1.0-Gy-irradiated-infected mice at 4 and 8 h, and 7.0-Gy-irradiated-infected mice at 30 h. Each lane indicates mRNA transcript from an individual mouse.
sham-irradiated-infected mice did not differ significantly across time, on the other hand in 7.0-Gy-irradiated-infected mice, relative IL-1\(\beta\) mRNA rose at 24 h and remained higher than those in sham II controls through 48 h. Significant differences (p < 0.05) from sham II controls (n = 6) were observed for 1.0-Gy-irradiated-infected mice at 24 h and sham-irradiated-infected mice at 4 h (Fig. 5A). A representative fluorograph of RT-PCR-amplified mRNA transcripts of IL-1\(\beta\) and GAPDH in spleens of sham II controls, sham-irradiated-infected mice at 4 h and 7.0-Gy-irradiated-infected mice at 24, 30 and 48 h is shown in Fig. 5B.

The increases in relative IL-6 mRNA after \(K.\ pneumoniae\) challenge occurred in a biphasic pattern (Fig. 6A) in all treatments. The first increase occurred in all irradiated and sham-irradiated mice at 4 h after bacterial challenge. Relative IL-6 mRNA decreased at 8 h in both 1.0-Gy- and 7.0-Gy-irradiated-infected mice. The appearance of the second increase depended on the dose of irradiation, occurring at 24–30 h after 7.0-Gy-infected mice, at 30–48 h after 1.0 Gy-infected mice, and at 48 h in the sham-irradiated-infected mice. A representative fluorograph of RT-PCR-amplified mRNA transcripts of IL-6 and GAPDH in spleens of sham II controls, sham-irradiated-infected mice at 4 h, 1.0-Gy-irradiated-infected mice at 4 h and 48 h, and 7.0-Gy-irradiated-infected mice at 24 and 30 h is shown in Fig. 6B.

Relative TNF-\(\alpha\) mRNA in 7.0-Gy-irradiated-infected mice were initially different from those of 1.0-Gy- and sham-irradiated-infected mice (Fig. 7A). Relative TNF-\(\alpha\) mRNA in the 7.0-Gy-irradiated-infected mice decreased slightly below the sham II control level at 1 and 4 h, while responses in 1.0-Gy- and sham-irradiated-infected mice were initially elevated at 1, 4 and 8 h, then declined to normal levels. There were no significant differences in responses between bacteria-challenged mice that were given 1.0 Gy and sham-irradiation. Significant differences (p < 0.05) from the sham II controls (n = 6) were observed in \(K.\ pneumoniae\)-challenged mice after 7.0 Gy at 30 h (1.6-fold increase); after 1.0 Gy at 4 and 8 h (2.0- and 2.1-fold increases); and after sham-irradiation at 1 h (2.1-fold increase). A representative fluorograph of RT-PCR-amplified mRNA transcripts of TNF-\(\alpha\) and GAPDH in spleens of sham II controls, sham-irradiated-infected mice at 1 h, 1.0-Gy-irradiated-infected mice at 4 h and 8 h, and 7.0-Gy-irradiated-infected mice at 30 h is shown in Fig. 7B.

**DISCUSSION**

This pioneering study demonstrated changes in cytokine gene expression in spleens of mice in response to sublethal gamma radiation and bacterial infection. \(K.\ pneumoniae\) is a virulent pathogen that induces an impressive inflammatory response in mice\(^{18}\). The initiation, maintenance, and eventual resolution of inflammation as a response to bacterial challenge depend on the expression of a complex network of both pro-inflammatory and anti-inflammatory cytokines. Elevated levels of TNF-\(\alpha\), macrophage inflammatory protein-2, and IL-10 in lung have been observed during \(K.\ pneumoniae\) infection\(^{19}\).

In this present paper we used the RT-PCR and Southern blot hybridization techniques to detect alterations in cytokine mRNA in spleens of mice after acute sublethal irradiation and in sublethally irradiated mice given a bacterial challenge. We report that the responses of se-
lected cytokines to the infectious challenge varied in patterns that depended upon the dose of radiation. However, the extent of the change in mRNA differed with time among various cytokines, suggesting that the combination of radiation and bacterial challenge produced different effects on cytokine mRNA transcription in the mouse.

Our studies showed a consistent trend for slightly elevated relative GM-CSF mRNA at days 4 and 7 after 7.0 Gy (Fig. 1A). During the equivalent period at 4, 8 and 24 hr in 7.0 Gy-irradiated-infected mice (time points located between vertical dashed lines in Fig. 1A), GM-CSF mRNA responses were significantly higher than those in the sham II control values (p < 0.05) (Fig. 4A). However, GM-CSF mRNA in sham-irradiated mice or mice given 1.0 Gy were similar at 4 h or longer following bacterial challenge. Bacterial challenge therefore appears to further increase the GM-CSF response induced by radiation alone. Along these lines, it was shown that GM-CSF-deficient mice had a propensity to develop lung and soft-tissue infection, and also had a significantly impaired long-term survival\(^2\). However, Basu et al\(^2\) showed that GM-CSF-deficient mice (a mix of C57BL/6 and 129/OLA strains) had a survival advantage with 58% of animals surviving at 50 hr as compared with 0% in the wild type following 500 \(\mu\)g LPS i.v. treatment on day zero. Along these lines, we have shown repeatedly that 7.0-Gy-irradiated mice do not survive challenge with *K. pneumoniae*\(^2\).

Several investigators detected an elevated accumulation of IL-1\(\beta\) in whole spleen after exposure to ionizing radiation. Nemoto et al\(^2\) demonstrated that IL-1\(\beta\) mRNA increased at 5–7 days after 3 Gy X rays in C3H/He male mouse. The increase of IL-1\(\beta\) at 3 Gy is less than 2–3 times the level at time zero. In our laboratory, the LD\(_{50/30}\) for B6D2F1/J female mouse is 9.0 Gy, whereas the LD\(_{50/30}\) for C3H/He male mice is 7.32 Gy. Further, X rays share a higher relative biological effect than \(\gamma\)Co gamma rays. Ishihara et al\(^2\) showed that immediate accumulation of IL-1\(\beta\) mRNA was found 30 min after the initiation of supra-lethal irradiation with 18.5 Gy X rays in C3H/HeN mouse and the increased level of message was not decreased until 4 h. In our results, relative IL-1\(\beta\) mRNA in 7.0-Gy-irradiated-infected mice decreased during the first day, then increased during the second day, but were elevated in sham-irradiated-infected mice during the first 4 h (Fig. 5A). This finding suggests that sublethal irradiation inhibits the increase of IL-1\(\beta\) gene transcription and leads directly or indirectly to increased susceptibility to infection. Therefore, IL-1\(\beta\) may have an important role in promoting innate resistance to bacterial infection in irradiated mice. In support of this, Peterson et al\(^2\) found that two bacterial derived immunomodulators, synthetic trehalose dicorynomycolate (STDCM) and an extract from *Serratia marcescens* (Sm-BRM) increased resistance to *K. pneumoniae* infection and also elevated gene expression of IL-1\(\beta\) in the spleen for 5 days after 7.0 Gy \(\gamma\) radiation. Along these lines, prophylactic administration of a low dose of recombinant IL-1\(\beta\) to mice 24 h before *Pseudomonas aeruginosa* infection increased the natural resistance to infection in granulocytopenic mice\(^2\).

Changes in relative IL-6 mRNA occurred in a biphasic manner in different treatment groups at different times (Fig. 6A). That is, the biphasic pattern became more delayed with increasing dose of radiation. Relative IL-6 mRNA did not significantly decrease during the first hour after infection, but increased in sham-irradiated-infected mice at 4 h. Relative IL-6 mRNA decreased 8 h after bacterial challenge, then increased the next day in 1.0-Gy- and 7.0-
Gy-irradiated-infected mice, but not in sham-irradiated-infected mice. Rapid alteration of IL-6 mRNA after infection is possible. Bacteria can induce a particular cytokine network, or alter an existing one, by stimulating the release of a variety of cytokines from host cells\textsuperscript{26}. Waage et al\textsuperscript{27} demonstrated that after administration of meningococcal (\textit{Neisseria meningitidis}) LPS to the subarachnoid space of rabbits, IL-6 concentration rose in the cerebrospinal fluid within 2 h, peaked after 3 h and declined to baseline after 9 h. Rola-Pleszczynski et al\textsuperscript{28} demonstrated that Leukotriene (LTB) stimulated IL-6 production and that underlying mechanisms involved both increased IL-6 gene transcription and message stabilization. This may constitute an important mechanism through which rapidly produced mediators may modulate the subsequent production of regulatory or growth-promoting cytokines. Along these lines, Peterson et al\textsuperscript{22} observed that S-TDCM and Sm-BRM treatments of 7.0-Gy-irradiated mice elevated splenic IL-6 mRNA after 3 days. Elevation of IL-6 protein was observed in spleen cells from \textit{Mycobacterium avium}-infected C57BL/6 mice at 2 weeks\textsuperscript{29} and in the circulation and spleens of mice infected with \textit{Staphylococcus aureus} at 24 h\textsuperscript{30}. Some investigators have considered persistently elevated IL-6 to predict mortality\textsuperscript{31}. However, Patchen et al\textsuperscript{32} showed that therapeutically administered IL-6 can effectively accelerate multilineage hematopoietic recovery following radiation-induced hematopoietic injury. Also, Neta et al\textsuperscript{33} demonstrated that interaction of IL-6 with IL-1 and TNF is a prerequisite for protection from radiation lethality.

In our studies relative TNF-\(\alpha\) mRNA remained essentially normal in irradiated mice (Fig. 3A). In contrast, relative TNF-\(\alpha\) mRNA was slightly below control values within 4 hr after bacterial challenge in 7.0-Gy-irradiated mice, whereas they were immediately elevated in 1.0-Gy- and sham-irradiated-infected mice (Fig. 7A). \textit{K. pneumoniae} challenge promoted elevation of relative TNF-\(\alpha\) mRNA in 1.0-Gy-irradiated-infected mice at 1–8 h and at 1–4 h in sham-irradiated-infected mice. Further, Peterson et al\textsuperscript{22} found that TNF-\(\alpha\) mRNA remained below normal levels 3–7 days after 7.0 Gy. Thus, elevation of TNF-\(\alpha\) mRNA in 7.0-Gy-irradiated mice by \textit{K. pneumoniae} appears to be delayed a few hours, after an initial depression. Increases in TNF-\(\alpha\) mRNA after bacterial infection has been associated with septic shock and pathogenesis of infectious diseases, but, if regulated, TNF-\(\alpha\) may provide the host a beneficial response to infection\textsuperscript{34}.

Peterson et al\textsuperscript{22} also demonstrated that S-TDCM and Sm-BRM enhanced survival from \textit{K. pneumoniae} challenge in irradiated mice. In mice treated with these immunomodulators, elevated IL-1\(\beta\) and IL-6 mRNA together with a low TNF-\(\alpha\) mRNA at the time of bacterial challenge appeared to be associated with survival from subsequent bacterial infection in irradiated mice. In contrast, our results showed that IL-1\(\beta\), IL-6 and TNF-\(\alpha\) mRNA remained near control values 1–8 h after \textit{K. pneumoniae} challenge in 7.0-Gy-irradiated mice. It would be appropriate, therefore, to examine the cytokine response that promotes resistance to infection in irradiated mice by determining cytokine gene expression in spleens of mice that are irradiated, treated with a nonspecific immunomodulator, known to enhance survival from infection, and then challenged with bacteria.

Mice given sublethal doses (e.g., 7.0 Gy) of gamma radiation are more susceptible to fatal bacterial infection than unirradiated mice\textsuperscript{35}. Thus, small doses of bacteria (e.g., \(1.8 \times 10^2\) CFU \textit{K. pneumoniae}) will cause death in 7.0-Gy-irradiated mice, whereas significantly larger
doses of bacteria (e.g., $7.5 \times 10^6 \text{ CFU } K. pneumoniae$) are required to achieve equivalent mortality in sham-irradiated mice (Fig. 8). Yet, there is little difference in mortality between 1.0-Gy and sham-irradiated mice.

The changes we measured in proinflammatory cytokine gene expression in irradiated mice indicate a possible relationship with increasing susceptibility to infection. A slightly delayed and prolonged increase in transcription of TNF-α and GM-CSF genes associated with biphasic responses of IL-6 mRNA indicates a poor prognosis for survival in acutely irradiated and infected animals. In contrast, elevated IL-1 mRNA coupled with a suppressed TNF-α mRNA response indicates a favorable prognosis due to enhanced resistance to infection. This concept needs further testing.

The effect of radiation on cells may be due to either direct interaction of the radiation with target molecules or indirectly through the generation of free radicals and oxidizing and reducing chemical species. Molecular damage caused by radiation is expressed through alteration of biochemical processes and is amplified by biological mediators. Cytokines, one type of mediators, are signaling molecules which can behave as classic endocrine hormones, but are better known for their autocrine and paracrine behavior, acting as integrating signals over short cellular distances. It is now realized that cytokines rarely, if ever, act in isolation but rather induce or inhibit other cytokines, thus, creating a population, or network, of cytokines to which cells respond. Ibuki et al reported that the activation of macrophages by radiation is caused by interaction with neighboring cells, such as lymphocytes, and by paracrine induction of certain cytokines which is initiated by the small amount of IL-1β released by irradiated macrophages. In the current paradigm, cytokine induction as a protective or pathological mechanism, is a direct response to the presence of infectious microorganisms.

![Fig. 8. LD$_{50/30}$ for K. pneumoniae in $^{60}$Co gamma-irradiated B6D2F1/J female mice (95% confidence limits).](https://academic.oup.com/jrr/article-abstract/41/3/259/1013713)
It is now clear that bacteria contain and produce a large number of diverse molecules, which can selectively induce the synthesis of both pro-inflammatory and immunomodulatory/anti-inflammatory cytokines\(^3\). Cytokines play an essential role in orchestrating the host’s response to radiation, infection, trauma, and other forms of injury. No work has been reported on gene expression and their associated proteins in irradiated animals with an induced infection. In previous studies, administration of recombinant cytokines as well as neutralizing antibodies to cytokines, established that IL-1 synergizes with TNF, CSFs, and IL-6 to protect mice from hematopoietic failure. In contrast, cytokines such as transforming growth factor (TGF)-\(\beta\) and interferon-gamma (IFN-\(\gamma\)) sensitize untreated mice to radiation lethality\(^6\). Thus, individual cytokines can be either beneficial or detrimental to the host’s recovery from damage due to ionizing radiation. An understanding of the signal transmitted to target cells by one or more cytokines and the context in which they operate will provide insight into the regulation of hematopoiesis, inflammation, wound closure (i.e., healing), and sepsis in irradiated animals. A better understanding may also lead to improved strategies for management of infection\(^3\). Studies of bacterial infection, particularly sepsis, require intricate physiological interactions among cells, tissues and organs. Resistance to infection depends upon a complex network of interacting signaling systems that involve the innate and adaptive immune systems, nervous system, and endocrine system. The physiology of these interacting networks is altered by radiation. The effects and responses, therefore, must be examined in whole, integrated animals, rather than limited cell or tissue cultures. Cell culture can be used as an analytic tool, but cell cultures cannot substitute for the complex interactions in an intact animal that provides the integrated biochemical, physiological, cellular, microbial, and multi-organ interactions necessary to perform these studies. The extrapolation of data from \textit{in vitro} cell or tissue cultures to accurately describe even a single facet of the immune response of the whole animal is difficult in studies designed to determine the effects of various treatments, such as ionizing radiation or drugs, on infection. Only in living, whole animals can the interaction of all the functioning components of the immune system be examined. It is therefore necessary to perform studies of the combined effects of ionizing radiation and bacteria in living experimental animals.

The data presented here provide impetus for subsequent studies on the role of proinflammatory cytokines in the pathogenesis of bacterial challenge in irradiated mice. We are concurrently determining cytokine proteins in spleen and plasma by using the ELISA method in another aspect of this study. These studies could lead to improved evaluation of the status of animals during the course of infection and efficacy of therapeutic interventions. Determining optimal cytokine responses for resistance to bacterial infection will aid in the development of potential therapeutic strategies for infection after radiation injury.

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