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Influence of Sublethal Total-Body Irradiation on Immune Cell Populations in the Intestinal Mucosa

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Abstract

The intestinal immune system is the largest in the body. This study analyzed changes in intestinal immune cell populations, cytokine protein levels, and transcript profiles after total-body irradiation (TBI) in CD2F1 mice. A single dose of 8.0 Gy γ radiation caused negligible 30-day lethality but induced significant histological damage in jejunal mucosa that was maximal at 3.5 days and that had seemingly recovered by day 21 after irradiation. These changes were accompanied by decreased numbers of mucosal macrophages, neutrophils, and B and T lymphocytes, mostly coinciding with similar reductions in peripheral blood cell counts. Recovery of mucosal macrophages occurred within 1 week, whereas mucosal granulocytes and lymphocytes remained low until 3 weeks after TBI. Maximal suppression of T-helper cell (T_H)-related transcripts occurred at 3.5 days, but there was no obvious T_H1 or T_H2 bias. Genomewide transcriptional profiling revealed a preponderance of differentially regulated genes involved in cell cycle control, cell death and DNA repair between 4 h and 3.5 days after irradiation. Genes involved in tissue recovery predominated from day 7 onward. We conclude that the intestinal immune system undergoes profound changes after sublethal TBI and that these changes likely contribute to postirradiation pathophysiological manifestations.

INTRODUCTION

The extents of injury to the bone marrow and the gastrointestinal tract are the principal determinants of survival after exposure to total-body irradiation (TBI). The terms “bone marrow” and “gastrointestinal” death (or syndrome) are generally used to refer to the predominant injury at various radiation doses and are based on the assumption that cell death in a single target (progenitor) cell compartment is responsible for injury. However, while convenient, these concepts do not adequately account for important interactions among organ systems or for the fact that multiple cell types in a given organ may influence the pathophysiology of radiation toxicity. The latter is particularly true for immune systems at locations other than in the bone marrow.

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SUPPLEMENTARY INFORMATION

Supplementary Tables S1–S3, Supplementary Figs. S1 and S2. <http://dx.doi.org/10.1667/RR1742.1.S1>

The intestinal mucosa harbors the largest and one of the most complex immune systems in the body. Moreover, even during normal conditions, the intestinal mucosa exhibits a state of “physiological inflammation”, manifested by the presence of abundant leukocytes in intraepithelial and subepithelial compartments (1). This is largely the consequence of an immune response to dietary and bacterial antigens that are present in the intestinal lumen (2). In situations where the epithelial barrier is compromised, such as after localized intestinal irradiation, immune cells are activated and inflammatory cell recruitment and transmigration increase by several orders of magnitude (3). Activated immune cells, primarily neutrophils, macrophages and cytotoxic T cells, attack and destroy neighboring cells either directly or indirectly through the release of soluble factors such as reactive oxygen and nitrogen metabolites, cytotoxic proteins, lytic enzymes or cytokines (1,4).

Changes in immune cell populations and accompanying changes in the expression levels of cytokines, chemokines and transcription factors after localized and abdominal irradiation have been reported by others (5-7). However, less is known about changes that occur in immune cell populations of the intestinal mucosa after TBI, when recruitment of cells from outside the irradiated field is influenced substantially by the development of systemic immune suppression. Moreover, the significance of these changes in terms of intestinal gene expression patterns and for conversion of the bowel into a “pro-inflammatory” organ is largely unknown. This study examined the temporal changes in intestinal immune cell populations and relevant cytokine transcript and protein levels as well as genome-wide comparative expression levels after exposure to a sublethal total-body dose of radiation.

The studies showed that the recovery of intestinal macrophages occurred early (within 1 week), whereas the mucosal populations of granulocytes and B and T lymphocytes remained low until 3 weeks postirradiation. Transcriptional profiling revealed a preponderance of differentially regulated genes involved in cell cycle control, cell death and DNA replication and repair during the early postirradiation period (4 h to 3.5 days), whereas genes representing pathways involved in tissue recovery and connective tissue development predominated from day 7 onward. These findings are relevant to the understanding of biological interactions in the gut mucosa after total-body exposure to radiation, to establishing a role for the intestine as an immunologically active organ, and to the development of strategies to mitigate or treat intestinal radiation toxicity.

MATERIALS AND METHODS

Animals

The experimental protocol was reviewed and approved by the University of Arkansas for Medical Sciences’ and the Central Arkansas Veterans Healthcare System’s (CAVHS) Institutional Animal Care and Use Committees (IACUC).

Experiments were carried out in a total of 76 random-bred male CD2F1 mice (Harlan Sprague Dawley, Indianapolis, IN) that were 6–7 weeks old with an initial body weight of 22–25 g. Animals were housed in conventional cages under standardized conditions with controlled temperature and humidity and a 12–12-h day-night light cycle. Animals had free access to water and chow (Harlan Teklad laboratory diet 7012, Purina Mills, St. Louis, MO).

Mice were exposed to radiation at a total-body single dose of 8.0 Gy. In previous experiments in CD2F1 mice, we showed that 8.0 Gy induces substantial intestinal and hematopoietic injury, but with adequate 30-day survival rates (8). Groups of 8–10 mice were killed humanely at set times after irradiation [0 h (no irradiation), 4 h, 1 day, 3.5 days, 7 days, 14 days, 21 days and 30 days]. Samples from individual mice were analyzed throughout, i.e., without pooling.

Irradiation

TBI was performed as described before (8,9). Briefly, after confirmation of dose uniformity by thermoluminescence dosimetry, irradiation was performed with a Shepherd Mark I, model 25, ¹³⁷Cs irradiator (J. L. Shepherd & Associates, San Fernando, CA). During irradiation, the animals were held in well-ventilated custom-made plexiglass restrainers on a turntable rotating at 5 revolutions per minute. The average dose rate was 1.35 Gy per minute.

Quantitative Immunohistochemistry and Image Analysis

For immunohistochemical studies, proximal segments of jejunum (10–15 cm from the ligament of Treitz) were obtained at 0 h (baseline), 4 h and 1, 3.5, 7, 14, 21 and 30 days, fixed in methanol-Carnoy's solution (60% methanol, 30% chloroform, 10% acetic acid), and embedded in paraffin to obtain 5-μm cross sections. The jejunum was chosen because it exhibits the most consistent radiation response and because of the paucity of Peyer's patches. The methanol-Carnoy's fixative, in contrast to formalin, is as good as frozen sections for immunohistochemical detection of leukocyte antigens (10).

Immunohistochemical staining for myeloperoxidase (MPO, neutrophils), macrophages, CD45R (B lymphocytes) and CD4 (CD4⁺ T lymphocytes) was performed with mouse-specific antibodies with methods established and optimized in our laboratory. Sections were deparaffinized and rehydrated. Endogenous peroxidase was blocked with 1% H₂O₂ in methanol for 30 min at room temperature. Nonspecific binding was reduced with 10% normal goat or 10% normal rabbit serum (Vector Laboratories, Burlingame, CA) in 3% dry powdered milk in Tris-buffered saline (TBS) for 30 min. Sections were incubated with rabbit anti-MPO (1:100, Dako, Glostrup, Denmark) or rat monoclonal anti-macrophage (RM0029-11H3), rat anti-CD45R, or rat anti-CD4 (all from Abcam, Cambridge, MA at 1:100) for 2 h. This was followed by a 30-min incubation with the following biotinylated antibodies: goat anti-rabbit IgG (MPO, 1:400, Vector Laboratories) and rabbit anti-rat IgG (for all other antibodies, 1:400, Abcam). Sections were further incubated with avidin-biotinperoxidase complex for 45 min (1:100, Vector Laboratories). Peroxidase binding was visualized with 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride solution (Sigma-Aldrich, St. Louis, MO) and 0.003% H₂O₂ in TBS.

Quantitative assessment of immunoreactivity was performed using computerized image analysis (Image-Pro Plus, Media Cybernetics, Silver Springs, MD) as described and validated before (11). Cells positive for MPO, macrophage antigen, CD45R and CD4 were identified by color thresholding. The number of positive cells per 10 fields (40× objective) was considered a single value for statistical analysis.

Preparation of Intestinal Tissue Homogenates

To prepare intestinal homogenates for protein analysis, tissues obtained at each time were homogenized in 600 μl of PBS supplemented with a complete protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN) and 160 U/ml RNase inhibitor (Promega, Madison, WI) using a polytron homogenizer (Brinkmann Instruments, Delran, NJ). Subsequently, homogenates were centrifuged at 14,000 rpm for 15 min at 4°C to pellet membrane material and supernatants were stored at −80°C until subjected to multiplex cytokine microbead array analysis as described below.

Multi-analyte Microbead Array to Detect Proinflammatory Mediator Expression

To assess inflammatory mediators in whole tissue homogenates of the proximal jejunum, a mouse 20-plex cytokine microbead array system was used according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). This microbead array allows for the simultaneous

measurement of the following 20 inflammatory molecules in a single 50- μ l sample: fibroblast growth factor 2 (FGF-2), GM-CSF, interferon γ (IFN- γ), IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 p40/p70, IL-13, IL-17, interferon-inducible protein 10 (IP-10), C-X-C motif ligand (CXCL)1/KC, monokine induced by IFN- γ (MIG, CXCL-9), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1 α (MIP-1 α), TNF- α and vascular endothelial growth factor α (VEGF- α). Results were analyzed using a Bioplex Workstation (Bio-Rad Laboratories, Hercules, CA) and normalized against the amount of total protein extracted from the intestinal tissues. The level of sensitivity for each microbead cytokine standard curve ranged from 1 to 40 pg/ml.

Blood Cell Counts

Whole blood was collected into EDTA-coated tubes (Fisher Scientific). Peripheral blood cell counts were obtained using a veterinary hemocytometer (Hematrue System, Heska Corporation, Loveland, CO) according to the manufacturer's instructions.

RNA Isolation

Steady-state mRNA levels were measured in jejunum tissue collected at different times after irradiation. Tissues were snap frozen in liquid nitrogen and stored at -80°C . Frozen tissue samples were homogenized in Ultraspec RNA reagent (Biotecx Laboratories, Houston, TX) according to the manufacturer's instructions. Two micrograms of RNA was kept for microarray analysis (including quality control analysis) and 2 μ g was used for real-time PCR.

Real-Time PCR

For real-time PCR, 2 μ g of RNA from whole intestinal tissues was treated with RQ-DNase I (Promega, Madison, WI) at 37°C for 30 min, after which cDNA was synthesized using a cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Steady-state mRNA levels were measured with real-time quantitative PCR (Taqman) using the following Applied Biosystems predesigned Taqman gene expression assays: IFN- γ , Mm00801778_m1; TNF- α , Mm00443258_m1; IL-4, Mm00445259_m1; IL-6, Mm00446190_m1; IL-33, Mm01195786_m1; CXCR3, Mm00438259_m1; CCR4, Mm00438271_m1; T-box 21, Mm00450960_m1; GATA-3, Mm00484683_m1; SOCS1, Mm00782550_s1; SOCS3, Mm00545913_s1; CCL9, Mm00441260_m1; CCL11, Mm00441238_m1; CCL5, Mm01302428_m1; and 18s rRNA, Hs99999901_s1. PCR amplification and detection were carried out on an ABI prism 7000 Detection System (Applied Biosystems). mRNA levels were normalized to eukaryotic 18s rRNA and calculated relative to control, unirradiated jejunum, using the standard $\Delta\Delta C_t$ method.

Focused T_H1-T_H2-T_H3 PCR Array

To determine possible time-dependent changes in T_H polarization in the jejunum after total-body irradiation, RNA from mouse intestine was analyzed using a focused mouse T_H1-T_H2-T_H3 PCR array that consisted of a panel of 84 transcripts (RT² Profiler PCR Array, SABiosciences, Frederick, MD), of which 24 were T_H1-related and 34 were T_H2-related. Reverse transcription, first-strand cDNA synthesis, and PCR reactions were performed by the service core facility of SABiosciences. All RNA samples ($n = 4$ per time) were tested for integrity and minimal degradation using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). One microgram of total RNA was reverse transcribed using an RT² First Strand kit. PCR reactions were performed on the ABI Fast 7900, using the PAMM-034 array (Mouse T_H1-T_H2-T_H3 PCR array) with RT² Real-Time SYBR Green PCR master mix. mRNA levels were normalized to the means of the following five housekeeping genes—Gusb, HPRT1, Hsp90ab1, GAPDH and ACTB—and relative changes were calculated relative to control, unirradiated jejunum using the $2^{-\Delta\Delta C_t}$ method.

Illumina Arrays

Microarrays were used to determine time-dependent changes in gene expression profiles in the proximal jejunum after TBI. Both generation of aRNA and microarray hybridization were performed by the Microarray Core Laboratory of the University of Texas Health Science Center (Houston, TX). All RNA samples ($n = 4/\text{time}$) were tested for integrity and minimal degradation using an Agilent Bioanalyzer 2100. Two hundred nanograms of total RNA was reverse transcribed and amplified overnight with T7 RNA polymerase and labeled with biotin following the manufacturer's protocol, in which 1.5 μg of biotin labeled aRNA was hybridized to microarrays at 58°C overnight. Illumina Whole Genome BeadChips (Mouse Ref⁶ v1, Illumina, San Diego, CA) representing approximately 46,000 mouse transcripts were used. Arrays were incubated with Cy3 streptavidin and washed according to the manufacturer's protocol.

Initial analysis of the microarray data was done using Illumina's Bead studio V1. After background subtraction, arrays were normalized to each other by quantile normalization. Changes in gene expression were tested using a modified t test that employs estimates of variation that include sequence-specific biological variation (σ_{bio}), non-specific biological variation (σ_{neg}), and technical error (σ_{tech}), according to the Illumina User Guide (2005), rev. B. Genes were considered differentially regulated at a P value less than 0.001. The open source clustering software Cluster (12) was used to perform hierarchical clustering of the five times, based on average gene expression values at each times. Differentially expressed genes were categorized according to cellular function using the Ingenuity Pathways Analysis software (Ingenuity Systems, Inc. Redwood City, CA). The life science search engine NextBio (NextBio, Cupertino, CA) was used to detect gene expression array data sets that show a high correlation with the 874 highest differentially expressed genes at 3.5 days after irradiation.

Statistical Analysis

Statistical analysis was done with the software package Number Cruncher Statistical Systems 2000 (NCSS) using one-way analysis of variance (ANOVA) followed by a two-sided Dunnett's multiple comparisons *post hoc* test for control specimens relative to samples from the other times. Pairwise data comparisons were performed with the Student's t test. A P value less than 0.05 was considered statistically significant. Data are reported as averages $\pm$ SEM.

RESULTS

Effects of Total-Body Irradiation on Peripheral Blood Counts

The 8-Gy radiation dose was generally well tolerated up to 30 days after irradiation, with only two mice dying on day 29. Tissues from these two animals were not included in the present analyses.

Radiation induced a significant decrease in the numbers of circulating lymphocytes, monocytes and granulocytes (Supplementary Information, Data, Fig. S1A). Reductions were observed as early as 4 h after irradiation. By day 30, monocyte counts were back to baseline levels and granulocyte counts were increased to above baseline ($P < 0.0004$). Thrombocyte counts were significantly reduced on days 7–21 ($P < 0.0001$) (Supplementary Information, Fig. S1B). Erythrocytes were significantly reduced only at 14 days after irradiation ($P < 0.0002$) and returned to baseline levels at 21 days (Supplementary Information, Fig. S1C).

Effects of Total-Body Irradiation on Immune Cell Populations in Jejunum Mucosa

As expected, the most pronounced structural changes in the intestinal mucosa were found at 3.5 and 7 days after irradiation, while the histology at the other times (4 h, 1 day and 21 days) was near normal (Supplementary Information, Fig. S2).

Immunohistochemistry was used to demonstrate the distribution of various types of immune cells in the intestinal mucosa. A time-dependent decrease in MPO-positive cells (mainly neutrophils) was noted that reached significance ($P < 0.01$) at 3.5 days and persisted until 21 days ($P < 0.001$). MPO-positive cells returned to baseline levels by day 30 (Fig. 1A). While a significant reduction in the number of macrophages was observed between 4 h and 3.5 days after irradiation, macrophages were increased from 14 days onward ($P < 0.005$) (Fig. 1B). CD45R-positive cells (B cells) and CD4-positive cells (mainly T-helper lymphocytes) were also reduced at earlier times up to 7 days after irradiation (Fig. 1C, D). Interestingly, by day 14 (i.e., at a time when peripheral blood lymphocyte counts were still low), the numbers started to increase, and baseline levels were achieved by day 21.

Radiation-Induced Alterations in Cytokines and Chemokines in Intestinal Tissue

A mouse 20-plex cytokine microbead assay was used to assess the levels of inflammatory mediator proteins in whole tissue homogenates of the proximal jejunum. Both TNF- α and MIG were significantly induced 3.5 days postirradiation (Fig. 2A, B). In contrast, significant attenuation of MIP-1 α was observed from 24 h–3.5 days ($P < 0.03$) and of IL-6 at 24 h ($P < 0.05$) (Fig. 2C, D). The mouse 20-plex assay contains two angiogenesis-related factors, FGF-2 and VEGF- α . FGF-2 exhibited a time-dependent increase with significant induction at 24 h and 3.5 days postirradiation and returned to baseline levels by day 21 (Fig. 3A). Tissue levels of VEGF- α were significantly reduced at 24 h ($P < 0.04$), after which an increase was noted from 3.5 days–21 days (Fig. 3B). IL-12 was highly attenuated from 24 h onward, with levels staying below the detection limit at these times. Both IL-4 and IFN- γ remained below the detection limit of the assay in both control and irradiated samples.

Effects of Total-Body Irradiation on CD4⁺ T Lymphocytes in Jejunum

T_H1/T_H2 gene expression patterns in the jejunum tissue were analyzed by quantitative real-time RT-PCR (Figs. 4 and 5). Since activated T-helper lymphocytes acquire and maintain a specific pattern of chemokine receptors, CXCR3 was used as a marker of T_H1. The CXCR3 levels in brain were about 20-fold lower than in intestine, while the levels in thymus were sixfold higher (Supplementary Information, Table S1). Radiation-induced repression of CXCR3 mRNA levels in the jejunum was noted as early as 4 h postirradiation and was maintained until 21 days ($P < 0.004$) (Fig. 4A). Likewise, the transcription factor T-Bet, which is selectively expressed in T_H1 cells, was significantly down-regulated after irradiation (Fig. 4B). When, in the presence of IL-12, activated T-helper lymphocytes differentiate into T_H1 cells, they secrete predominantly IFN- γ and IL-2. As shown in Fig. 4C, however, the mRNA level of IFN- γ was not altered by radiation. Further, radiation did not induce any alterations in TNF- α and SOCS1 mRNA levels (Fig. 4D, E).

Radiation significantly induced the mRNA levels of IL-6, CCL9 and SOCS3. IL-6 and CCL9 (macrophage inflammatory protein 1 γ) were both significantly induced at 3.5 days postirradiation ($P < 0.002$ and $P < 0.001$) (Fig. 5A, B). SOCS3 exhibited a time-dependent increase with a significant induction by day 21 postirradiation ($P < 0.03$, Fig. 5C). In contrast, the relatively T_H2 selective transcription factor GATA-3 was repressed after irradiation (Fig. 5D). The relative mRNA levels of IL-4 and CCR4 also were not significantly different from controls (Fig. 5E, F).

Analysis of the data from the focused array provided generally similar results. Of the 24 T_H1-related transcripts, 12 were differentially expressed relative to baseline at one or more times, while 19 of the 34 T_H2-related transcripts were differentially expressed. At 4 h, 24 h and 3.5 days, all of the differentially expressed T_H1-related genes and a majority of the T_H2-related genes were down-regulated. In contrast, the majority of differentially expressed genes (both

T_H1- and T_H2-related) were up-regulated at 7 and 21 days (Supplementary Information, Table S2).

Of the key T_H1-related genes, IFN- γ was not significantly altered at any of the times examined. A significantly decreased expression of IL-2, one of the other T_H1 cytokines representing a major growth factor for the expansion of activated T cells, was noted at 3.5 days after irradiation. A significant down-regulation was also noted for CD28 CXCR3, IGSF6, IL18, IL-27ra, T-bet, Stat1 and CD40 at this time. However, both SOCS1 and Stat1 expression were significantly up-regulated by day 21 post irradiation.

TBI significantly induced the mRNA levels of IL-10, CEBPB and JAK3 as early as 4–24 h postirradiation, whereas IL4ra, JunB, c-MAF and SOCS3 were up-regulated by day 21 postirradiation. On the other hand, IL-4, which drives T_H2 development, remained unaltered compared to controls after irradiation and GATA-3, a lineage-specific factor selectively expressed in the T_H2 pathway, was significantly down-regulated as early as 24 h postirradiation.

Overall, the T-helper cell response clearly exhibited a time-dependent course with profound changes relative to baseline, but there was no clear bias toward a T_H1 or T_H2 response.

Genome-Wide Analysis of Transcript Levels in the Jejunum

Microarrays containing approximately 46,000 mouse probes were used to compare gene expression profiles in the jejunum at 0 h, 4 h, 24 h, 3.5 days, 7 days and 21 days. Between 21,645 and 22,564 probes were detected at each time. Of these probes, a total of 162 were differentially expressed at 4 h after irradiation. Similarly, a total of 225, 1071, 149 and 205 probes were differentially expressed at 24 h, 3.5 days, 7 days and 21 days, respectively.

Quantitative real-time RT-PCR was used to verify results obtained with microarrays. *Ccl9* was found to be significantly up-regulated at 3.5 days in microarray analysis and was verified by RT-PCR (relative mRNA: 3.75 ± 0.06 , $P < 0.001$). In addition, down-regulation of *Ccl5* at 3.5, 7 and 21 days was verified by RT-PCR (relative mRNAs: 0.40 ± 0.04 , 0.39 ± 0.01 and 0.22 ± 0.14 , respectively). Similarly, down-regulation *Ccl11* and *Il33* was found at 3.5 days after irradiation, and these alterations were confirmed by RT-PCR (relative mRNAs: 0.72 ± 0.08 and 0.40 ± 0.08 , respectively).

Hierarchical clustering of the five times was performed, based on the average gene expression values obtained from microarrays at each time (Fig. 6A). The 7-day and 21-day times clustered together, as well as the 4-h and 24-h times, while the average gene expression profile at 3.5 days differed more from those at all other times.

Figure 6B shows cellular function-based gene categories with the largest proportion of differential expression at 3.5 days after irradiation. Gastrointestinal disease and DNA repair and replication were among the most affected gene categories at this time. Table 1 gives an overview of highly altered gene categories at each time. A large proportion of the genes that were differentially expressed up to 3.5 days after irradiation were involved in cell cycle regulation and cell death, while genes representing pathways involved in tissue regeneration and connective tissue development predominated at 7 and 21 days.

With regard to the immune system, 275 probes involved in inflammation or immune response were identified. Twenty-seven of these probes were differentially expressed at one or more times after irradiation (Supplementary Information, Table S3). Twenty of these 27 probes were differentially expressed at 3.5 days. Genes that were differentially expressed at more than one time were *Cd8b1* (coding for CD8 beta chain 1, down-regulated from 24 h onward), *Ccl5*

(down-regulated from 3.5 days onward), *C4a* (complement component 4a, up-regulated at 24 h and 3.5 days), and *Reg3g* (regenerating islet-derived III γ , up-regulated at 3.5 and 7 days). The microarrays represented at least 60 Ig heavy chain gene segments. Thirty-one of these showed high average expression values in controls (>100 fluorescence units) and were significantly down-regulated between 4 h and 7 days after irradiation, coinciding with reduced numbers of mucosal B cells at these times. Similarly, six T-cell receptor segments were found of which two, *Tcrb-V8.2* and *Tcra*, were down-regulated at all times after irradiation. Neutrophil-specific genes, including *C177*, *Ela2*, *Mmp25* and *Mpo*, showed a low average expression signal (< 100) throughout the experiment. Although some macrophage-specific genes, including *Mpeg1*, *Mgl2* and *Mmd*, displayed reduced transcript levels between 4 h and 3.5 days after irradiation and increased levels at 21 days, these changes were not significant.

DISCUSSION

The intestinal mucosa represents one of the most complex parts of the immune system, and the gut is frequently referred to as the largest lymphoid organ of the body (2,13). Thus gut-associated lymphoid tissue (GALT), which is intimately associated with the gut epithelium, constitutes the largest mass of immune cells that function in antigen-specific immune responses (14).

An inflammatory reaction is a classical feature of radiation exposure and is presumed to play a pivotal role in the development of both acute and delayed radiation responses in many tissues. However, while changes in intestinal immune cell populations after localized and abdominal irradiation have been reported (5-7), comparatively little is known about alterations that occur after TBI, when recruitment of cells from outside the irradiated field is substantially influenced by systemic immune suppression.

The findings that the various intestinal immune cell populations exhibited profound changes and that these changes, with some notable exceptions, paralleled changes in the peripheral blood are not surprising.

Our study demonstrated a reduction in mucosal macrophages as early as 4 h after irradiation. Little is known about radiation-induced killing of monocyte/macrophage lineage cells. While earlier reports suggested that mature macrophages in the bone marrow, lymph nodes and peritoneal cavity are relatively radioresistant (15), more recent studies suggest that mature as well as immature macrophages are radiosensitive (16).

Results from the present study suggest that B lymphocytes were somewhat more radiosensitive than T lymphocytes, which agrees with other reports (13,17,18). However, interestingly, mucosal lymphocytes (both B and T lymphocytes) appeared to recover before the recovery of the circulating lymphocyte count. This finding may indicate that the proliferation of surviving lymphocytes in the intestinal mucosa is particularly active and/or that circulating lymphocytes home specifically to the bowel mucosa after being depleted after TBI.

The cytokine environment plays an important role in influencing T_H development (19). Some reports suggested that ionizing radiation induces the preferential differentiation of T_H cells into T_{H2} cells in spleen (20,21) and more recently in intestine after localized or abdominal irradiation (6,7). In the present study, a combination of cytokine analysis using a multibead (Luminex) platform, RT-PCR, and a focused T_H-cell PCR array was used to assess T_H cell bias. With the caveat that the results of analyses of steady-state mRNA and protein levels in complex tissues do not necessarily reflect transcript levels in specific cell types and that various other cell types may express T-cell "specific" transcripts (22-25), the present study did not reveal an obvious T_{H1} or T_{H2} bias at any of the times studied up to 30 days after TBI. It is conceivable that the lack of T_H cell polarization in the present study reflects either differences

between localized/abdominal irradiation and TBI or the known differences among species and strains within the same species in terms of T_H1 or T_H2 dominance. Further studies to address radiation-induced changes in T_H1-T_H2 cells and other T-cell subsets that may play important roles in the intestine such as $\gamma\delta$ T cells and IL-10-producing T_{reg} cells are clearly warranted.

Transcriptional profiling with microarrays and subsequent pathway analysis revealed a prevalence of differentially expressed genes involved in pathways related to cell cycle, cell death, DNA replication and DNA repair at times up to 3.5 days after irradiation. From day 7 onward, pathways related to connective tissue development, gastrointestinal disease and muscular development predominated, consistent with the notion that the intestine entered a phase of tissue repair at this time. Differentially expressed genes also belonged to the categories cancer and reproductive system disease, which is a reflection of genes involved in cell cycle control and cell death.

Specific gene expression profiles mostly correlated with numbers of inflammatory cells in the mucosa. While neutrophil-specific genes showed low average expression levels at all times, some macrophage-derived genes showed a trend of expression correlating with numbers of macrophages at the different times after irradiation. Finally, expression profiles of Ig heavy-chain genes correlated with the numbers of mucosal B cells, and T-cell receptor genes correlated with the numbers of mucosal T lymphocytes after irradiation. Specifically with regard to genes involved in inflammation or immune response, 275 probes were identified on the microarrays. These probes represent genes that were differentially expressed at more than one time after irradiation, including CD8 beta1, which was down-regulated from 24 h onward, and CCL5, a chemokine produced by CD8⁺ T lymphocytes, which was down-regulated from 3.5 days onward. Indeed, studies have shown a marked decrease in the number of CD8⁺ cells in the intestine after irradiation (26). C4a, on the other hand, was up-regulated at 24 h and 3.5 days. This complement component may be cleaved into c4 anaphylatoxin, a mediator of local inflammation. In addition, regenerating islet-derived III γ was up-regulated at 3.5 and 7 days. This mediator is a C-type lectin with bactericidal properties that is stored in Paneth cell granules and increased by inflammatory stimuli and mucosal damage (27-29).

In conclusion, temporal alterations in mucosal immune cells and cytokines after a sublethal dose of radiation point to the relevance of the intestine as an immunologically active organ that likely plays an active role in the systemic radiation response. These findings may be important for future development of strategies to mitigate or treat intestinal radiation toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIG. 1.

Effects of total-body irradiation on the numbers of intestinal immune cells. Radiation induced a time-dependent decrease in the numbers of MPO-positive cells (mainly neutrophils). By day 30, the numbers of MPO-positive cells returned to baseline level (panel A). Radiation induced a significant reduction in numbers of macrophages up to 3.5 days and an increase from 14 days onward (panel B). Radiation reduced numbers of CD45R-positive cells (B cells) (panel C) and CD4-positive cells (panel D). Ten fields per section with a 40 $\times$ objective. Averages + SEM, $n = 4-6$. $F = F$ ratio; $DF =$ degrees of freedom. Heavy horizontal lines indicate significant difference compared to the 0-h (control) group by Dunnett's multiple range test.

FIG. 2.

Effects of total-body irradiation on inflammatory mediators and chemokines in whole tissue homogenates of the jejunum. Radiation induced a significant increase in TNF- α at 3.5 day (panel A) and induced significant increases in MIG at 3.5 and 21 days (panel B). MIP-1 α was significantly reduced at 24 h and 3.5 days and reached baseline levels at 21 days (panel C). Similarly, IL-6 was significantly reduced on 24 h and reached baseline levels at 21 days (panel D). Averages + SEM, $n = 5$. Abbreviations and symbols as for Fig. 1.

FIG. 3.

Effects of total-body irradiation on angiogenesis-related mediators in whole tissue homogenates of the jejunum. FGF-2 exhibited a time-dependent increase with significant inductions at 24 h and 3.5 days (panel A). VEGF- α was significantly reduced at 24 h and reached baseline levels by 7–21 days after irradiation (panel B). Averages + SEM, $n = 5$. Abbreviations and symbols as for Fig. 1.

FIG. 4.

Effects of total-body irradiation on Th1-related genes in the jejunum. Radiation significantly reduced mRNA levels of CXCR3 (panel A) and T-bet (panel B) but did not alter mRNA levels of IFN- γ (panel C), TNF- α (panel D) or SOCS1 (panel E). Averages + SEM, $n = 4-6$. Differences are indicated only for transcripts where the ANOVA showed an overall significant difference. Abbreviations and symbols as for Fig. 1.

FIG. 5.

Effects of total-body irradiation on Th2-related genes in the jejunum. Radiation significantly induced mRNA levels of IL-6 (panel A), CCL-9 (panel B), and SOCS3 (panel C), while mRNA levels of GATA-3 were reduced (panel D). Relative mRNA levels of IL-4 and CCR4 at each individual time were not significantly different from controls at a significance level of 0.05 (panels E, F). Average + SEM, $n = 4-6$. Abbreviations and symbols as for Fig. 3.

FIG. 6.

Example of gene expression array results. Hierarchical clustering of average gene expression profiles revealed that the profiles of the first two times (4 h and 24 h) and the last two times (7 days and 21 days) clustered together (panel A). Ingenuity software was used to classify 3.5-day differentially expressed genes according to cellular function. Gastrointestinal disease and DNA replication and repair were among the most altered categories at this times (panel B). $n = 4$.

TABLE 1

Most-Altered Gene Categories According to Cellular Function

Cellular function	4 h	24 h	3.5 days	7 days	21 days
Cancer	6.1	11	21.7	9.8	9.4
Cell death	6.1	8.5	5.8		4.4
Cell cycle	5.2	4.6	6.3		3.1
Cell assembly and organization	3.8	4.4	7.5	3.8	3.3
Cellular growth and proliferation	5.1	5.8	4.2		3.8
DNA replication, recombination and repair		3.8	7.5	3.5	
Connective tissue development and function	3.1	4.2		6.5	3.7
Skeletal and muscular system development and function		4.2	3.8	6.5	3.7
Gastrointestinal disease			10		
Immunological disease	5.6		3.3		4.8
Hematological disease	5.5	4.8		3.3	4.9
Reproductive system disease	3.4	5.0	7.5	7.3	6.3

Notes. The negative logarithm of the P value (−log(P value)) is given for each time after irradiation. All values >6 (i.e., $P < 10^{-6}$) are shown in bold.