The Analysis of the Defense Mechanism against Indigenous Bacterial Translocation in X-Irradiated Mice

Toshiya Kobayashi,*1 Toshihiro Ohmori,1 Minoru Yanai,1 Gosei Kawanishi,1 Masao Mitsuyama,2 and Kikuo Nomoto3

1Life Science Research Institute, Snow Brand Milk Products, Tochigi 329-05, 2Department of Bacteriology, Niigata University School of Medicine, Niigata, Niigata 951, and 3Department of Immunology, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Fukuoka 812

(Accepted for publication, February 8, 1991)

Abstract The defense mechanism against indigenous bacterial translocation was studied using a model of endogenous infection in X-irradiated mice. All mice irradiated with 9 Gy died from day 8 to day 15 after irradiation. The death of mice was observed in parallel with the appearance of bacteria from day 7 in various organs, and the causative agent was identified to be *Escherichia coli*, an indigenous bacterium translocating from the intestine. Decrease in the number of blood leukocytes, peritoneal cells and lymphocytes in Peyer’s patches or mesenteric lymph nodes was observed as early as 1 day after irradiation with 6 or 9 Gy. The mitogenic response of lymphocytes from various lymphoid tissues was severely affected as well. The impairment of these parameters for host defense reached the peak 3 days after irradiation and there was no recovery. However, *in vivo* bactericidal activity of Kupffer cells in mice irradiated with 9 Gy was maintained in a normal level for a longer period. It was suggested that Kupffer cells play an important role in the defense against indigenous bacteria translocating from the intestine in mice.

Whole-body exposure of mammals to ionizing radiation produces varying degrees of acute injury depending upon the dose of irradiation. In a dose of lethal range, death follows as a result of damage to the central nervous system, gut injury, or bone marrow failure accompanied by a loss of resistance to bacterial infection. The range above 200 Gy has been regarded as “brain death range,” that from 10 to 200 Gy as “gut death range” and that from 5 to 10 Gy as “hematopoietic death range” (6).

The hematopoietic death is fundamentally due to bone marrow failure, but the direct cause of this death is considered to be bacterial infection. The bacterial infection appears to be endogenous infection caused by translocation of the indigenous bacteria which inhabit the intestinal tract (5, 8, 9, 12). The defect of host defense mechanism resulting from bone marrow failure seems to be responsible for the translocation of the bacteria (3, 9).

In the present study, we have attempted to clarify the mechanism involved in the prevention of bacterial translocation using a model of endogenous infection in X-irradiated mice.
MATERIALS AND METHODS

Animals. Specific pathogen-free female ICR mice (7–9 weeks old) were obtained from Charles River Japan, Inc. (Atsugi, Kanagawa, Japan). All mice were housed under barrier-sustained conditions in our animal facility.

X-irradiation. Mice were exposed to 6 or 9 Gy of whole-body X-irradiation. The radiation was delivered from a Hitachi X-irradiation machine MBR-1505R (Hitachi Medico Co., Ltd., Tokyo) operating at 0.2 Gy/min with 1.0 mm Al and 0.2 mm Cu filtration.

Bacterial counts in feces and various organs. The numbers of viable bacteria in feces, peripheral blood, liver, spleen, lung, peritoneal cavity and mesenteric lymph node (MLN) were determined on various days after 9 Gy irradiation. Feces were collected through anus. Peripheral blood was obtained by puncture of retro-orbital venous plexus. Then mice were completely bled to death. The peritoneal washing fluid was obtained by lavaging the peritoneal cavity with 5 ml of sterile phosphate-buffered saline (PBS). Then the liver, spleen, lung and MLN were removed and homogenized in 10 ml of PBS using a Teflon homogenizer. The homogenates were diluted serially with PBS and spread on the trypticase soy agar plates. Plates were incubated aerobically at 37°C for 24 hr, and colonies on the plate were enumerated.

Identification of translocating bacteria. The bacteria which translocated to the liver, spleen and lung was identified on day 11 after 9 Gy irradiation. The media used for identification of bacteria were as follows: (i) Enterobacteriaceae, MacConkey agar (Nissui Seiyaku Co., Ltd., Tokyo); (ii) Staphylococci, Mannitol-salt agar (Nissui Seiyaku); (iii) total anaerobes, GAM agar (Nissui Seiyaku); (iv) Lactobacilli, LBS agar (BBL Microbiology Systems, Cockeysville, Md., U.S.A.) supplemented with 0.8% Lab lemco powder (Oxoid Ltd., Basingstoke, U.K.), 0.15% sodium acetate trihydrate and 0.37% acetate. All anaerobic plates were incubated anaerobically using Gas Pak system (BBL Microbiology Systems) at 37°C for 72 hr, and aerobic plates were incubated aerobically at 37°C for 24 hr. Species and biotypes of Enterobacteriaceae were identified using Biotest No. 1 (Eiken Kagaku Co., Ltd., Tokyo).

Histopathological examination of small intestines. Mice were killed by cervical dislocation after 9 Gy irradiation and small intestines were removed. Histopathological examination was performed by hematoxylin-eosin staining after formalin fixation.

Blood cell counts and leukocyte analysis. Peripheral blood was obtained by puncture of retro-orbital venous plexus using a heparinized Pasteur pipet on various days after 6 Gy irradiation. White blood cells (WBC), red blood cells (RBC) and platelets (PLT) were counted using a Sysmex blood cell auto counter E-4000 (Toa Medical Electronics Co., Ltd., Kobe, Japan). The dose of irradiation, 6 Gy, was chosen in this experiment to examine the mice for a longer period after irradiation. Smear samples of peripheral blood were examined for the differential count of neutrophil, lymphocyte, monocyte, eosinophil and basophil.

Spleen and thymus weights and bone marrow cell counts. Spleen, thymus and femur were removed on various days after 6 Gy irradiation. Weights of spleen and thymus
were measured. Bone marrow cells were prepared by repeated flushing of femur with RPMI 1640 medium using a 1 ml syringe with a 26-gauge needle, and the cell number was counted microscopically.

**Counts of Peyer's patches and cells from Peyer's patches.** Mice were killed on various days after 9 Gy irradiation. The number of Peyer's patches in an entire small intestine was counted macroscopically. Then Peyer's patches were cut out from intestine and cut into small pieces. Cells were dispersed by mashing the pieces of Peyer's patches held between a pair of frosted microslide glasses and suspended in RPMI 1640 medium, followed by passing the cells through #150 and #80 stainless meshes. The number of total cells was counted microscopically.

**Proliferative response of lymphoid cells to mitogens.** The proliferative response of lymphoid cells in the spleen, thymus and Peyer's patches to lipopolysaccharide (LPS) and concanavalin A (Con A) was determined by tritiated thymidine incorporation. Cells were suspended to give $2 \times 10^6$ cells/ml in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and fetal calf serum (10%). Cells were cultured in the presence of LPS (5 or 25 µg/ml) or Con A (1 or 5 µg/ml) in 96-well flat-bottom microplate for 48 hr at 37°C in a humidified atmosphere of 5% CO$_2$ and air. Cultured cells were harvested 15 hr after the pulse with tritiated thymidine. Incorporated radioactivity was counted using a Beckman scintillation counter LS-9800 (Beckman Instruments Inc., Fullerton, U.S.A.).

**Counts of peritoneal exudate cells and phagocytic activity of peritoneal macrophages.** Peritoneal exudate cells (PEC) induced by an intraperitoneal injection of 5 ml of 10% proteose peptone 3 days before 9 Gy irradiation were washed out of the peritoneal cavity with Hanks' balanced salt solution (HBSS) on various days after irradiation. The cells were counted microscopically. After centrifugation at 200 × g for 5 min, the cell pellet was resuspended at $2 \times 10^6$ cells/ml in HBSS and placed in a 60 mm culture dish containing a sterilized cover glass. After incubation for 2 hr at 37°C in humidity atmosphere of 5% CO$_2$ and air, dishes were washed with warm HBSS three times to remove nonadherent cells. One milliliter of *Listeria monocytogenes* suspension ($5 \times 10^7$ cfu/ml) was added to adherent cells and incubated for 1 hr in the same condition as above. After washing with HBSS three times, the cover glass was removed, fixed by methanol and stained with Giemsa's solution. The number of bacteria phagocytosed by macrophages was counted microscopically and expressed as the mean number per 20 macrophages.

**Activity of Kupffer cells for bacterial killing in vivo.** Mice were inoculated intravenously with streptomycin-resistant *Escherichia coli* (*E. coli* K12 ML1410) on various days after 9 Gy irradiation. Mice were sacrificed by cervical dislocation 10 min and 4 hr after the inoculation. Liver was removed and homogenized in 10 ml of PBS with a Teflon homogenizer. Then the homogenate was diluted serially and spread on trypticase soy agar plates containing streptomycin sulfate (100 µg/ml), which inhibited the growth of indigenous bacteria but not that of inoculated bacteria. Plates were incubated at 37°C for 24 hr and colonies grown on the plates were counted. The difference in the colony counts between at 10 min and 4 hr was regarded as the bacterial killing by Kupffer cells *in vivo*. 
RESULTS

Survival of Mice and Appearance of Bacteria in Various Organs after X-Irradiation

Survival of mice irradiated with 9 Gy was observed every day. Mice died from 8 to 15 days after irradiation. The numbers of viable bacteria in feces and various organs were determined on various days after 9 Gy irradiation. The fecal bacterial counts began to increase from day 5 and reached a level about 100 times as high as those of normal mice by day 11. Until day 5, no bacteria was recovered from any organ. Bacteria became detectable in peripheral blood, spleen, liver and lung from day 7, and in peritoneal cavity and MLN from day 9 after irradiation (Fig. 1).

Identification of the Translocating Bacteria

The bacteria recovered from the liver, spleen and lung formed uniform colonies on MacConkey and GAM agar plate, suggesting that they are facultative anaerobes and belonging to Enterobacteriaceae. Species of the bacteria was identified as Escherichia coli from the results of bacteriological examination using Biotest No. 1.

Histopathological Changes of Small Intestine

No desquamation of villus epithelial cells or hemorrhage in submucosa was observed until day 8 when some mice began to die. Only a slight degree of atrophy was observed in intestinal villi after irradiation. On the other hand, a conspicuous

![Fig. 1. Kinetic change of bacterial number in various organs after 9 Gy irradiation. Each symbol represents the mean of 3 mice. ◊, feces (g); ○, liver; □, lung; △, spleen; ●, blood (ml); ■, mesenteric lymph node; ▲, peritoneal cavity.](image)
Fig. 2. Kinetic change of systemic immune system after 6 Gy irradiation. Each symbol represents the mean of 5 mice. ○, white blood cells; □, bone marrow cells; △, thymus weight; ●, spleen weight; ■, platelets; ▲, red blood cells.

Fig. 3. Composition of white blood cells. Each symbol represents the mean of 3 mice. ■, neutrophils; □, lymphocytes; □, monocytes.
necrosis of lymph nodules was observed in the section obtained immediately after irradiation.

**Effect of X-Irradiation on Blood Cells, Bone Marrow Cells and Weights of Thymus and Spleen**

By day 1 after 6 Gy irradiation, a significant decrease was observed in numbers of peripheral WBC and bone marrow cells and weights of thymus and spleen. All the values reached less than 20% of control level by day 3 and there was no recovery during observation for 14 days. A gradual decrease was observed in the number of RBC or PLT in peripheral blood (Fig. 2). Differential analysis of peripheral WBC revealed that the number of lymphocytes decreased immediately after irradiation, while the number of neutrophils was maintained in a control level until day 1 after irradiation (Fig. 3).

**Effect of X-Irradiation on Lymphoid Tissues**

The total number of Peyer's patches countable in intestine decreased as early as 1 day after irradiation and there was a complete loss of macroscopically detectable Peyer's patches on day 5. Total cell number was significantly decreased in both

---

**Table 1. Kinetic change in gut-associated lymphoid tissues after irradiation**

<table>
<thead>
<tr>
<th>Before irradiation</th>
<th>Days after irradiation&lt;sup&gt;a)&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Peyer's patches</td>
<td>10.4&lt;sup&gt;b)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total cell number in Peyer's patches (×10&lt;sup&gt;6&lt;/sup&gt;)</td>
<td>6.7</td>
</tr>
<tr>
<td>Total cell number in mesenteric lymph node (×10&lt;sup&gt;6&lt;/sup&gt;)</td>
<td>18.0</td>
</tr>
</tbody>
</table>

<sup>a)</sup> Dose of irradiation was 9 Gy.
<sup>b)</sup> Mean of 3 mice.
<sup>c)</sup> n.c. = not countable.

---

**Table 2. Changes in the mitogenic response of lymphocytes after irradiation**

<table>
<thead>
<tr>
<th>Lymphocytes from</th>
<th>Irradiation&lt;sup&gt;a)&lt;/sup&gt;</th>
<th>[&lt;sup&gt;3&lt;/sup&gt;H]TdR incorporation (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Con A (µg/ml)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Spleen</td>
<td>(−)</td>
<td>2,500&lt;sup&gt;b)&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(+)</td>
<td>0</td>
</tr>
<tr>
<td>Thymus</td>
<td>(−)</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>(+)</td>
<td>0</td>
</tr>
<tr>
<td>Peyer's patch</td>
<td>(−)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(+)</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a)</sup> Dose of irradiation was 9 Gy.
<sup>b)</sup> Mean of 3 mice.
DEFENSE AGAINST BACTERIAL TRANSLOCATION

Table 3. Kinetic changes in the number of peritoneal exudate cells (PEC) and phagocytic activity of peritoneal macrophage after irradiation

<table>
<thead>
<tr>
<th>Days after irradiation</th>
<th>No. of PEC ( \times 10^6 )</th>
<th>Phagocytic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Irradiation (−)</td>
<td>Irradiation (+)</td>
</tr>
<tr>
<td>0.1</td>
<td>2.7(^b)</td>
<td>1.5</td>
</tr>
<tr>
<td>1</td>
<td>2.8</td>
<td>0.8</td>
</tr>
<tr>
<td>3</td>
<td>3.4</td>
<td>0.6</td>
</tr>
</tbody>
</table>

\( a\) Dose of irradiation was 9 Gy.
\( b\) Mean of 3 mice.
\( c\) Mean number of \( L. \) monocylogen\( e\) phagocytosed in 20 macrophages.

Effect of X-Irradiation on Cells of Macrophage Lineage

PEC were induced in mice by intraperitoneal injection of irritant 3 days before irradiation and mice were irradiated or left unirradiated on day 0. When the total number of recoverable PEC was examined, a significant decrease was observed after irradiation and it became quite difficult to obtain countable number of cells after day 4 (Table 3). In contrast, function of a given number of macrophages as determined by phagocytosis for \( L. \) monocylogen\( e\) remained in the control level even on day 3 after irradiation (Table 3). Further analysis on functional change could not be carried out after this time point because of the difficulty in harvesting a sufficient number of macrophages.

Table 4. Kinetic change in the activity of Kupffer cells for bacterial killing \( \text{in vivo} \) after irradiation

<table>
<thead>
<tr>
<th>Days after irradiation</th>
<th>Log(_{10}) No. of i.v. injected bacteria in liver</th>
<th>Killing activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 min after injection (x)</td>
<td>4 hr after injection (y)</td>
</tr>
<tr>
<td>Not treated</td>
<td>6.2</td>
<td>4.8</td>
</tr>
<tr>
<td>0.1</td>
<td>6.2</td>
<td>5.0</td>
</tr>
<tr>
<td>1</td>
<td>6.3</td>
<td>5.2</td>
</tr>
<tr>
<td>3</td>
<td>5.5</td>
<td>4.4</td>
</tr>
<tr>
<td>5</td>
<td>5.9</td>
<td>5.2</td>
</tr>
<tr>
<td>7</td>
<td>6.1</td>
<td>5.3</td>
</tr>
<tr>
<td>9</td>
<td>5.2</td>
<td>4.6</td>
</tr>
</tbody>
</table>

\( a\) Dose of irradiation was 9 Gy.
\( b\) Mean of 3 mice.
Next we examined the activity of Kupffer cells in the liver to kill intravenously injected *E. coli*. Decrease of the number of *E. coli* per whole liver in 4 hr was taken as the parameter for killing activity of Kupffer cells *in vivo*. In order to avoid the contamination of indigenous *E. coli* translocating at the later phase after irradiation, streptomycin-resistant *E. coli* was employed for inoculation. As shown in Table 4, there was no significant change in killing activity until day 3. A gradual decrease was observed in bacterial killing *in vivo* after day 5; however, over 50% of activity was maintained until day 7 when some mice began to die.

**DISCUSSION**

Although various species of bacteria inhabit the intestinal tract of mammals, extraintestinal invasion by these indigenous bacteria never occurs in normal condition. There appears to be a current consensus that some host defense mechanism is preventing extraintestinal invasion of indigenous intestinal bacteria.

Invasion of indigenous bacteria into extraintestinal space is termed "translocation" (1). There are several reports which indicate that translocation of indigenous bacteria is facilitated in experimental animals receiving various treatments, such as ionizing radiation (9), tumor-transplantation (11), administration of immuno-suppressive agents (2, 4) and thermal injury (7). All these conditions are known to induce the depression of host defense mechanism. However, it is not clear what sort of impairment in host defense is relevant to the translocation of indigenous bacteria in each condition.

The first defense mechanism against the xenobiotics is the physical surface barrier, such as skin and mucosal membrane, and nonspecific humoral factors covering these surfaces, such as complement, lysozyme and lactoferrin. In this respect, the first line of defense against indigenous bacteria may be the mucosal membrane of intestinal tract. Histopathological examination in the present study showed that intestinal mucosal membrane was hardly damaged by X-irradiation. Therefore, the death due to bacterial translocation seemed to be resulting from the depression of defense mechanisms other than the first line of defense.

The Peyer’s patches and MLN construct gut-associated lymphoid tissue. In Peyer’s patches, antigens are taken up and presented to T or B cells. The MLN is the place where T and B cells mature and proliferate. These tissues are considered to be the initial site of mucosal immune response and act as the barrier to indigenous bacteria. In this study, we observed that X-irradiation caused a rapid and profound decrease in the cellularity of Peyer’s patches and MLN. In addition, the function of lymphoid cells in Peyer’s patches as determined by mitogenic response was significantly depressed by X-irradiation. These results indicated that local immune system is quite rapidly and almost completely depressed by X-irradiation, while bacterial translocation is still blocked.

After X-irradiation with 6–9 Gy, a very rapid decrease was observed in the numbers of peripheral WBC and bone marrow cells, and in the weights of thymus and spleen as well. Among WBC, lymphocytes were the most sensitive to X-irradia-
tion and decreased immediately after irradiation, while neutrophils were relatively resistant to X-irradiation and remained in the normal level for up to 1 day after irradiation. The proliferating ability of spleen cells and thymocytes was impaired completely by X-irradiation. Peritoneal exudate cells decreased 1 day after irradiation, but the recovered macrophages showed a normal level of phagocytic activity \textit{in vitro} even after 3 days. Though different immunocompetent cells showed an apparently different susceptibility to X-irradiation, systemic immune system seemed to have been extremely damaged by X-irradiation with 6–9 Gy by day 3 after irradiation. If the immune system, which is extremely susceptible to irradiation, is normally operating defense system against bacterial translocation, endogenous infection would have been observed from day 3. However, bacterial translocation was not observed until 7 days after irradiation. There might be some other defense mechanism acting in the period between days 3 and 7.

Kupffer cells are fixed macrophages in the liver, capable of ingesting and killing bacteria. Our results showed that activity of Kupffer cells for bacterial killing remained unaffected until day 7. It is suggested that Kupffer cells are relatively resistant to X-irradiation and play an important role as the defense mechanism covering the depressed local and systemic immune system after X-irradiation for up to day 7.

In this study, a part of the defense mechanism against translocation of indigenous bacteria was clarified by the use of model of endogenous infection in irradiated mice. Other studies in different models including tumor-bearing mice or mice treated with immunosuppressive agents will be helpful for analyzing the defense mechanism against indigenous bacteria which do not translocate in normal mice.

REFERENCES


(Received for publication, January 7, 1991)