Experimental Approach to Improving Early Postirradiation Restoration in the Hemopoietic System of Irradiated Canines

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Acute radiation disease/Bone marrow manipulation/Radiation-injury modification/Postirradiation treatment/Postirradiation hemopoietic restoration.

Experiments on mongrel canines exposed to total body irradiation in free-in-air dose of 3.85 (LD 95/45) or 4.05 Gy were carried out to evaluate the therapeutic effectiveness of some of bone marrow immediate-after-irradiation extraction, incubation in the presence of protein synthesis inhibitor and reimplantation in the organism (the reimplantation method) or bone marrow extraction alone (the extraction method). There were tested various kinds of methods that differed in bone marrow-blood mix volume extracted, in large or in small volumes. It has been determined that the reimplantation method in large volumes and the extraction method in small volumes are equally effective. Being supplemented with supportive therapy by antibiotics during d8–d24, the methods allowed the rescue at 4.05 Gy irradiation significantly more animals than in the case of therapy alone or its combination with ineffective variants of the methods. The positive effect of the methods manifested in a higher level of leukocyte nadir on d17, earlier reaching 0.5 × 10⁹ and 1 × 10⁹ leukocytes per liter, and increasing 45 days survival. The mechanism of positive influence of the methods on the radiation injury of hemopoiesis seems to be related to increased cytokine producing because of the irritation of bone marrow stromal cells and thus favorable interference in the early restoration processes.

INTRODUCTION

There are some statements that enable a search for the improvement of radiation injury treatment: imperfection of postirradiation DNA repair processes,1,2 the presence of reserves in the stem hemopoietic compartment even after rather massive exposures,3,4 and postponed and inadequate-to-danger mobilizing some protective reactions by organism.5 It follows from these statements the need of urgent (immediate-after-irradiation) interference in the course of early postirradiation processes to alter the ratio of injury/restoration in favor of last. However, the medicinal treatment (nonsymptomatic) is absent now in the protocol of urgent help for victims of a radiation accident.9,10

The idea remains poorly studied and unrealized that early postirradiation repair processes in a mammalian organism could be significantly improved and/or accelerated. In experiment on microorganisms and cell cultures, the main features of postirradiation repair were studied comprehensively, and the approaches were found to reach more complete restoration. These approaches consisted in suboptimum conditions of cultivation during the few hours after irradiation or in the use of some inhibitors of protein and DNA synthesis.5,11-13 The main goal of such interference was an inhibition of replication DNA synthesis avoiding a restriction of the preparation one. However, the ideas were not realized even in the praxis of experimental therapy, let alone in the human clinics. One reason for such a state seems to be the absence of convenient experimental models for research. The model must answer the following requirements: to be rather simple, approximated at most to a human clinic, reliable, and giving the possibility of being manipulated with cells mostly important for a surviving organism.

Earlier we described a new experimental procedure for various mammalian species to research different possibilities of increasing hemopoietic stem cells repair initiated with acute ionizing radiation injury.14,15 The procedure consists of immediate postirradiation extraction of some of bone marrow, its short-term incubation under conditions chosen, and reimplantation in the organism. It was referred to as the reimplantation method or briefly the EIR. It has been shown that the EIR influences positively saving the hemopoietic stem poten-
tial and surviving irradiated mice.\textsuperscript{50} However, it turned out that the main impact of the procedure in the therapeutic effect gave the first stage of the EIR. That was why bone marrow extraction had been used as a means for separate treatment. It is referred to as the extraction method, or briefly the Ex.

Furthermore, some results of the experiments suggested that the injection of the incubated blood-bone marrow mix was not only returning any quantity of stem cells into the organism. There is a complex mutual interaction between the organism and returning material, including cells and humoral components. The interaction depends obviously on the composition of returning mix and on the interval between extraction and reimplantation, and it can be as synergistic as antagonistic.

Further, we decided to make the model more approximated to a human clinics. For this reason we used canines in our experiments. The technology of bone marrow extraction underwent changes, because only spongy, not tubular, canine bones contain red bone marrow. The aspiration of blood-bone marrow mix instead of the flushing of a tubular bone had been done. That is why we needed to examine the effectiveness of the modified procedures. We tried also to examine the role of various experimental conditions in therapeutic effectiveness of both the reimplantation method and the extraction method. The results of the experiments are presented in this article.

\textbf{MATERIALS AND METHODS}

\textbf{Animals}

Canine holding, care, handling, and experimental procedures were approved by the management of the State Research Centre–Institute of Biophysics. Research was conducted according to \textit{Common Regulations on Standard Conditions of Experiments Carrying Out on Animals in The Institute of Biophysics of The Ministry of Health}.

Healthy male and female canines (mongrels 1–5 years old and weighing 10–20 kg) had been used in these studies. The canines were delivered from the Russian Academy of Sciences laboratory animal nursery after being immunized against distemper, hepatitis, enteritis, and rabies and treated to eliminate helminths. The canines were housed in individual cages. They were fed natural provisions that included all the main ingredients in proportions approved by the Ministry of Agriculture. Water was provided ad libitum. The canines were in the open air once a day.

They were observed for at least 1–1.5 months to be examined and selected for an experiment on the basis of such assays as blood leukocyte number (ranging in 6–12 \times 10^9 liter\textsuperscript{-1}), erythrocyte sedimentation rate (no more than 10 mm Hg), rectal temperature (no more than 39.5\degree C), weight kinetics (absence of decreasing), and stool (absence of any signs of diarrhea).

\textbf{Irradiation}

The dosimetry measurements had been done according to principles established for large animals by V. Bond and coauthors.\textsuperscript{61} The canines were exposed to multilateral irradiation from the \textsuperscript{60}Co-source EG0-2 in a plexiglas box at a dose rate of 3–4.8 mGy s\textsuperscript{-1}. The difference between free-in-air doses in the exposure space did not exceed 5%. Dosimetry measurements carried out earlier for the same source in air and on an appropriate phantom using ferrosulfate and ionizing method allowed the possibility of determining a tissue-air ratio of absorbed doses for canines of different weights.\textsuperscript{17} We used coefficients ranging from 0.73–0.82 to estimate exposures in terms of midline tissue doses. According to preliminary experiments, LD50 for mongrels exposed from the \textsuperscript{60}Co-source is calculated to be 2.93 Gy at midpoint free-in-air and in a range of 2.14–2.4 (an average 2.27) Gy at midline tissue for canines weighing 10–20 kg. The canines were exposed to 3.85 or 4.05 Gy free-in-air doses. The midline tissue doses for canines of different weights used were calculated to be in a range 2.81–3.16 (an average 2.98) Gy and 2.96–3.32 (an average 3.14) Gy, correspondingly.

\textbf{The procedures}

The scheme of the reimplantation method, including the extraction method, is presented in Fig. 1. A canine had been anesthetized 15–20 min after exposure by thiopental sodium 45 mg/kg IV or ketalin (Apharmo) 10 mg kg\textsuperscript{-1} IM and a few later with 5 mg kg\textsuperscript{-1} IV. Then we punctured spongy parts of bones with special needles with mandrines and extracted different volumes of blood-bone marrow mix in the presence of anticoagulants (heparin or sodium citrate in standard quantities).

The blood-bone marrow mix was placed in broad bowls, providing that the height of the liquid layer did not exceed 5 mm. There were added antibiotics and protein synthesis

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig1.png}
\caption{The scheme of the reimplantation method (EIR) including the extraction method (Ex) as the first stage of the EIR.}
\end{figure}
inhibitor cycloheximide (Sigma) at a concentration of 0.01 mg ml⁻¹. The bowls were kept 1–5 h in thermostat at 37°C. Then the mix underwent stirring, nuclear cell counting, and reimplanting via a leg vein in the organism.

According to the volume extracted, both procedures were divided as performed in large or small volumes. The abbreviations designating the procedures therefore have the marks “I” or “s.” The characteristics of the experimental groups formed on these grounds are presented in Table 1. The locations of punctures were as follows: iliac crest, sternum, and the upper metaphyses of tibia, femur, and humerus.

Supportive care

All canines, including the control, had been treated with systemic antibiotics from d8 to d24 or to the day when a leukocyte number reached 1 10⁹ liter⁻¹. Two antibiotics were administered intramuscularly twice daily: penicillin (0.5 g) or ampicillin (0.25 g) and streptomycin (0.25 g) or gentamycin (4% solution, 1.0 ml). Kanamycin was administered in tablets in case of diarrhea. Flushing of mouth cavity and throat by the antiseptic furacillin was carried out when signs of stomatitis or tonsillitis appeared. Dry furacillin was used in the event of any skin wounds or ulcers.

Examination

The clinical examination was performed every day. It included physical inspection to detect hemorrhages and skin damages, to evaluate appetite and state of mucous membranes, and to measure rectal temperature and weight (these two twice a week).

Hematological examination included peripheral blood counts twice a week. Blood was withdrawn from the subcutaneous vein of forelegs. Erythrocytes and leukocytes were counted microscopically by using the Goryaev count chamber. In specifically colored smears, we estimated thrombocyte and reticulocyte numbers on 10³ erythrocytes, leucocyte differentials, and we then calculated different blood cell counts per liter. Survival in experimental groups was estimated by d45. The day of each animal’s death within the examination period was recorded.

The efficacy of procedures were as follows: leukocyte nadir, the day of neutrophil number 0.5 10⁹ and 1 × 10⁹ per liter, 45 days survival.

RESULTS

Survival studies

The effectiveness of different variants of the procedures on assays of 45-day survival and mean survival time (MST) for those who died is presented in Table 2. The data show the advantage of the reimplantation method in large volumes (EIR-I) and the extraction method in small volumes (Ex-s) over two resting variants and the control group at a dose of 4.05 Gy. The absence of difference in survival between the same groups at a dose of 3.85 Gy seems to be a result of available effectiveness of the only supportive therapy. There is tendency toward a positive effect in increasing MST for the reimplantation method at both doses.

Statistical analyses of the survival data using Fisher variant of χ² confirms the suggestion that there is the significant positive influence of the EIR-I and the Ex-s on the acute radiation sickness outcome (Table 3). The difference in survival between 2 combined groups (control and ineffective treatment variants on the one hand and effective ones on the other) is valid for dose 4.05 Gy, as in the case of combining data for both doses (p = 0.02).

Postirradiation kinetics of different blood cells

The data of leukocyte and erythrocyte kinetics at either dose are combined for each of the experimental groups to have more representative data, and they are presented in Fig. 2.

Data for 2 canines with automyelotransplantation, AMT (bone marrow extraction proceeded irradiation by 2 h) are not presented in Fig. 2. They are shown below.

It follows from Fig. 2A that about equally effective variants of the procedures are the EIR-I and the Ex-s. Their curves lie above the curves for the EIR-s, the Ex-s and the control. Both treatment variants increase significantly the level of leukocyte nadir and accelerate the achievement of complete leukocyte number restoration. Two other variants lack such effectiveness. True, the uncertainty takes place regarding the EIR-s. This variant demonstrated the most speed of leukocyte resto-

Table 2. Canine survival (survived/tested) and mean survival time (MST) for dead in different experimental groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>3.85 Gy Survival</th>
<th>MST (days)</th>
<th>4.05 Gy Survival</th>
<th>MST (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reimplantation method</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EIR-1</td>
<td>2/3</td>
<td>24</td>
<td>3/5</td>
<td>21 ± 2.0</td>
</tr>
<tr>
<td>EIR-s</td>
<td>1/1</td>
<td>1/4</td>
<td>1/5</td>
<td>21 ± 3.5</td>
</tr>
<tr>
<td>Extraction method</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ex-l</td>
<td>1/3</td>
<td>25.5 ± 4.5</td>
<td>0/3</td>
<td>17.7 ± 1.8</td>
</tr>
<tr>
<td>Ex-s</td>
<td>1/1</td>
<td></td>
<td>4/6</td>
<td>18.5 ± 0.5</td>
</tr>
<tr>
<td>Control</td>
<td>2/3</td>
<td>17</td>
<td>1/6</td>
<td>17.4 ± 1.5</td>
</tr>
</tbody>
</table>

Mongrel canines were treated immediately after exposure with the reimplantation method (EIR, see Fig. 1) or with the extraction one (Ex) both in large (l) or in small (s) volume of bone marrow-blood mix, that was extracted. All canines, including controls, were treated with antibiotics within d8-d24.

Table 3. Statistical analysis of difference in surviving exposed (4.05 Gy) dogs between effective treatment variants, noneffective ones and control.

<table>
<thead>
<tr>
<th>Groups compared</th>
<th>Survived/tested</th>
<th>Difference (%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>EIR-1 + Ex-s</td>
<td>7/11</td>
<td>47</td>
<td>0.08</td>
</tr>
<tr>
<td>Control</td>
<td>1/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EIR-1 + Ex-s</td>
<td>7/11</td>
<td>49</td>
<td>0.05</td>
</tr>
<tr>
<td>EIR-s + Ex-l</td>
<td>1/7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EIR-1 + Ex-s</td>
<td>7/11</td>
<td>48</td>
<td>0.02</td>
</tr>
<tr>
<td>Control +</td>
<td>2/13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ EIR-s + Ex-l</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mongrel canines were irradiated with 4.05 Gy 90Co source. Immediately after exposure canines were treated with the reimplantation method (EIR) or with the extraction one (Ex) both in large or small volumes, meaning the volume of bone marrow-blood mix extracted. All canines, including controls, were treated with antibiotics within d8-d24.

In regard to erythrocyte kinetics in Fig. 2B, the advantage of the EIR-1 and the Ex-s is expressed in a lower degree over the others. Only the variant Ex-l is distinguished relative to others. The erythrocyte number falls dramatically after d17 in the case.

All the most informative points of leukocyte kinetics are presented in Table 4. These figures are obtained by means of their determination in every canine and the subsequent counting of an average of a statistical numerical row. The data in Table 4 show the significant difference in leukocyte nadir between the EIR-1 and the Ex-s on one hand, and each of the other procedure variants including the control on the other. However, according to test of the day of 0.5 × 10^9 or 1 × 10^9 leukocytes per liter all the treatment variants, especially AMT, demonstrate an advantage over the control. AMT certainly exceeds the effective procedure variants on all the leukocyte kinetics indexes, and to more extent on the time reaching the registration points of leukocyte numbers and on the nadir assay.

The extraction of bone marrow itself shows the tendency to increase the leukocyte abortive rise, whereas the reimplantation of incubated bone marrow seems to reduce the effect noticed. It is assumed to mean under the abortive rise the possible leukocyte number rise, staying in the period 7–10 postirradiation days that is followed by the repeated decrease of leukocyte.

Improving Restoration in Irradiated Canines

Table 4. Some indexes of post-irradiation leukocyte kinetics in canines exposed to 3.85 or 4.05 Gy (data combined) in different experimental groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Leukocyte nadir, (1 \times 10^5 ) at survivors</th>
<th>Day of leukocyte</th>
<th>Leukocyte ratio* d10/d7 (time of abortive rise%), %</th>
</tr>
</thead>
<tbody>
<tr>
<td>EIR-1</td>
<td>0.34 ± 0.07</td>
<td>22.1 ± 0.8</td>
<td>23.6 ± 0.9</td>
</tr>
<tr>
<td>EIR-s</td>
<td>0.2</td>
<td>18.0</td>
<td>19.2</td>
</tr>
<tr>
<td>Ex-I</td>
<td>0.1</td>
<td>22.9</td>
<td>23.6</td>
</tr>
<tr>
<td>Ex-s</td>
<td>0.39 ± 0.1</td>
<td>22.3 ± 1.4</td>
<td>23.5 ± 1.2</td>
</tr>
<tr>
<td>AMT</td>
<td>0.52 ± 0.12</td>
<td>13.2 ± 1.2</td>
<td>15.8 ± 1.2</td>
</tr>
<tr>
<td>Control</td>
<td>0.19 ± 0.006</td>
<td>26.2 ± 0.7</td>
<td>27.8 ± 1.0</td>
</tr>
</tbody>
</table>

*Leukocyte ratio d10/d7 is calculated as the ratio of leukocyte number on d10 to the one on d7. *Abortive rise means that the possible leukocyte rise in the period 7–10 postirradiation days is followed by the repeated leukocyte decreasing. *b.m. extraction 2 h before irradiation and returning 3 h later.

The postirradiation kinetics of basic leukocyte types and platelets for both effective treatment variants, AMT and the control, are presented in Fig. 3. Like common leukocytes, neutrophils and platelets demonstrate nadir rising for both procedures, but lymphocytes do so in a lesser degree. It can also be remarked that the tendency was first to more expression of a neutrophil abortive rise for both procedures and AMT in comparison to the control, second to postponed platelets number restoration in the EIR-1 compared with the Ex-s, and third to the most early beginning neutrophil number restoration for AMT in comparison with all the resting groups.

DISCUSSION

Earlier we showed that either of the procedures (EIR and Ex) is capable of positive influencing the course of acute radiation sickness and hemopoietic restoration in lethally and sublethally irradiated mice. Now the same result has been achieved in mongrel canines. In previous preliminary experiments on canines exposed to the free-in-air dose of 3.6 Gy, the EIR alone, without the subsequent supportive therapy, allowed to rescue from death 2 dogs from 5, whereas all 5 control dogs died. We then decided to check the potencies of the EIR and the Ex at more severe exposures. The dose of radiation exposure was elevated in beginning up to 3.85 Gy and eventually up to 4.05 Gy free-in-air being accompanied by antibiotics therapy in the acute period of the disease. The treatment of canines immediately after irradiation by an effective variant of both the reimplantation and the extraction method with the subsequent antibiotics therapy resulted in an earlier restoration of hemopoiesis and in higher survival than in the control group treated only with antibiotics. The positive effect had been observed regarding the kinetics of all the blood cell types and was especially pronounced for neutrophils. Thus the modification of the procedure EIR, i.e., bone marrow extraction from spongy bones in dogs instead of flushing tubular bones from mice did not alter the ability of
the procedure to influence positively postirradiation processes.

What lies at the base of the favorable action of effective variants of the procedures used, and what prevents such action in case of ineffective variants? In accordance with the literature6,18–20 and our previous experiments on mice, we can believe that the main acting factor is an irradiation of bone marrow by means of numerical bone punctures and blood-red bone marrow mixture extraction. It is obvious enough for the earlier and may be a more powerful initiation of restoration processes in a damaged hemopoietic system on the level of early progenitor cells. Some material for considering that topic gives Fig. 4. Besides our data, we also took for comparison the data of Storb and his coauthors.

The extrapolation of curves representing a restoration of neutrophil number to ordinate axis suggests the different level of precursors soon after exposure or the different time of beginning the restoration in the case of an equal level of precursors. Storb's data seem to prove the leading role of regeneration elevation, but not the repair activation, meaning under former dividing survived stem cells and under second the recovery of lethally injured stem cells. The single administration of either procedure (EIR in large volumes or Ex in small volumes) immediately after irradiation is sufficient for receiving the therapeutic effect in contrast with the need of numerical G-CSF injections during 3 weeks. This fact does not yet testify in favor of repair processes prevailing in the case of the procedure usage, but it suggests such a possibility.

The closeness of the therapeutic effects of the EIR-l and the Ex-s suggests a uselessness of the incubation–reimplantation stages. However, if to compare the effects of the reimplantation method and the extraction one, both in large volumes, it becomes obvious the therapeutic effect of the returned blood-

bone marrow mix (Fig. 2). True, it is not clear so far whether the positive effect takes place because of repaired stem cells, or due to the erythrocytes returned, or owing to some physiologically active substances including cytokines that could be produced during incubation.

There are also other arguments in favor of that the EIR procedure has more prospective worth. These are as follows: a more powerful irradiation action of the Ex-l compared with the Ex-s; receiving a bulk of exposed bone marrow for manipulations, namely, to use any survived stem cells for their expansion in culture and subsequent grafting or to try to repair injured stem cells. The last remains poorly studied so far, though it represents a tempting but difficult task.

Some other questions concern the influences of reimplanted blood-bone marrow mix on processes evoked in an organism by the mix extraction and vice versa. There is a suggestion that these mutual relations can be synergistic or antagonistic, depending on the size of bone marrow extraction and the time of incubation/reimplantation. That may be why we have such unexpected manifestations as an ineffectiveness of the EIR-l in contrast to the Ex-s, and absence of positive action of the EIR-l under conditions of 1 h mix incubation at 4°C (data are not shown). Rather like antagonism had been observed in the work concerned radioprotectors testing: combination of a lipopolysaccharide analog and 2 prostaglandins was ineffective in the radioprotection of lethally irradiated dogs.22 Both ingredients of the combination are synergistic on their radioprotection capacity, and the ineffectiveness of the combination looks like the situation with our ineffective variants of the procedures. Another common reason of impeding the radiotherapeutic effect of means like the EIR/Ex could consist in stress and an increased glucocorticoid level. So dexamethason reduced the radioprotective efficacy of inflammation evoked by turpentine oil.23

In the previous article,25 we supposed that the positive action of the procedures presented on postirradiation processes is due to the elevated production of some cytokines. The compare of the effectiveness of the postirradiation cytokine therapy and of the EIR-l/Ex-s regarding canines is presented in Fig. 5. A daily administration to beagles of G- or GM-CSF within d1–d21 exposed to TBI with supportive therapy in an acute period gave the effect of LD50 increasing in terms of DRF to be 1.7, according to McVittie et al.24 and 1.6 according to R. Storb et al.21 The effectiveness of the procedures EIR-l and Ex-s at their single administration immediately after exposure, including further subsequent supportive therapy, was evaluated to 1.5 on the same criterion regarding mongrel dogs. The closeness of the therapeutic effectiveness of the procedures, and cytokine therapy enables us to speculate about the cytokines involved. Taking into consideration the traumatic character of both procedures, it seems to be such cytokines as interleukin-l and the tumor necrosis factor.
Fig. 5. The comparison of the therapeutic effectiveness of Colony Stimulating Factors and the procedures EIR/EX regarding acute radiation sickness of animals. Data concerning CSFs are taken from the following works: Storb et al. 81: rh or rc G-CSF s.c. daily within d0–d20 after exposure; MacVitie et al. 24: rh G-CSF or rh GM-CSF s.c. daily within d1–d21/d24.

REFERENCES