Hematopoiesis in Long-Term Survival Following Supralethal Irradiation and Bone Marrow Transplantation in Dogs

By J. L. Chertkov, M. N. Novikova, N. M. Nemenova and V. N. Malanina

Bone marrow transplantation may effectively protect dogs exposed to whole-body ionizing radiations of 2 to 3 LD95. Hematopoiesis was investigated at remote periods after the exposure, when the hematopoietic stem cells had been proliferating in the lethally irradiated organism for a long time.

Ferrebee and his associates succeeded in attaining long-term survival of supralethally irradiated dogs given autografts of bone marrow or allografts and methotrexate. However, no details concerning bone marrow hematopoiesis in these animals were given. The only information was that 6 months to 4 years subsequent to exposure, the leukocyte, reticulocyte, and thrombocyte counts and the hemoglobin level in the peripheral blood were within normal ranges.

For a number of years this laboratory has been investigating the effect of hematopoietic cell transplantation in radiation lesions. It was shown in earlier works that usually bone marrow allografts have no positive effect on lethally irradiated dogs owing to the rapid transformation of the introduced hematopoietic cells into lymphocytes immunologically active against the recipient, a circumstance conducive to the cessation of normal hematopoiesis. The dogs expire within two weeks following exposure. In a number of cases we succeeded in preventing or inhibiting the lymphoid transformation of the transplanted allogeneic cells by resorting to transplantation of mixed bone marrow derived from several donors, delayed transplantation of the bone marrow, transplantation of filtered (through a plug of cotton) bone marrow, predominantly containing lymphoid cells and erythronormoblasts, etc. Several of the animals treated thus survived. On the other hand, a good protective effect was obtained by transplantation of bone marrow autografts. Thus we were in a position to investigate hematopoiesis at remote periods after exposure to two lethal doses of ionizing radiation in dogs protected by allogeneic or autologous hematopoietic cells.
Table 1.—Investigation of Irradiated Dogs Given Bone Marrow Transplantations

<table>
<thead>
<tr>
<th>Nos. of dogs</th>
<th>Transplanted hemopoietic tissue</th>
<th>Infusion of hemopoietic cells following irradiation (days)</th>
<th>Number of transplanted nucleate cells (x 10⁹)</th>
<th>Period of observation (days)</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>96</td>
<td>Allogeneic bone marrow</td>
<td>6</td>
<td>15.0</td>
<td>1155</td>
<td>Killed</td>
</tr>
<tr>
<td>101</td>
<td>Allogeneic lymphocytes</td>
<td>2</td>
<td>0.17</td>
<td>1030</td>
<td>&quot;</td>
</tr>
<tr>
<td>159</td>
<td>Allogeneic bone marrow</td>
<td>2</td>
<td>9.9</td>
<td>543</td>
<td>&quot;</td>
</tr>
<tr>
<td>161</td>
<td>Mixture of allogeneic bone marrow from 6 donors</td>
<td>2</td>
<td>4.5</td>
<td>496</td>
<td>&quot;</td>
</tr>
<tr>
<td>178</td>
<td>Allogeneic bone marrow filtered through a plug of cotton</td>
<td>2</td>
<td>3.9</td>
<td>436</td>
<td>Alive</td>
</tr>
<tr>
<td>181</td>
<td>Autologous bone marrow</td>
<td>1</td>
<td>4.1</td>
<td>232</td>
<td>Killed</td>
</tr>
<tr>
<td>188*</td>
<td>&quot;</td>
<td>1</td>
<td>15.6</td>
<td>151</td>
<td>&quot;</td>
</tr>
<tr>
<td>189*</td>
<td>&quot;</td>
<td>1</td>
<td>8.1</td>
<td>151</td>
<td>&quot;</td>
</tr>
<tr>
<td>206</td>
<td>&quot;</td>
<td>1</td>
<td>4.2</td>
<td>205</td>
<td>Alive</td>
</tr>
</tbody>
</table>

*Thymectomized dogs.

MATERIALS AND METHODS

Nine mongrel dogs of both sexes, aged from 2 to 6 years and weighing 12 to 15 Kg., were exposed to 1000 r. (Table 1). Five of the animals received protective grafts of allogeneic hematopoietic cells, four were grafted with autologous cells. The animals were exposed to simultaneous emissions from four RUM-3 units mounted on the left and right sides and top and front of the exposure chamber. The intensity of the doses conforming to the capacity of the chamber (80x30x40 cm.) oscillated within ± 10 per cent. Irradiation specifications: amperage 10 ma., voltage 195 kv., filters Cu 0.5 mm. and Al 1.0 mm., focal distance 50-60 cm., intensity of dose in atmosphere 20-25 r./min.

No anthelminthics were administered since no perceptible worm infestation was observed. The animals were kept in separate cages under normal conditions. Their daily ration was supplemented with 2 eggs and 250 Gm. of milk curds during the first twenty days following exposure. During this period they were also given daily intramuscular injections of penicillin (200,000 U) and streptomycin (100,000 U).

Morphological studies of the peripheral blood and of bone marrow punctates of all the dogs were made 2 or 3 times preceding irradiation and at regular intervals subsequently. Data recorded: erythrocyte, leukocyte, thrombocyte, and reticulocyte counts per 1 mm³ blood; Hb value, ESR, and myelokaryocyte count per 1 mm³ bone marrow. Blood and marrow smears were fixated in methanol and stained with azure II eosin.

The animals were killed by electrocution. Pieces of their organs removed for histologic examination were fixated in neutral formalin and embedded in paraffin and celloidin. The sections were stained by several methods: Van Gieson and hematoxylin-eosin, Pap silver impregnation, Perls, Sudan IV.

Autografts for transplantation were obtained directly preceding irradiation by aspiration from the heads of the humerus and femur, and from the iliac crest into culture medium 199 containing dry heparin (60 units per ml.). Usually 100-150 ml. of bone marrow was extracted from each animal (4.1 - 15.6 x 10⁹ nucleated cells) and mixed with 100 ml. of the medium. The bone marrow was refrigerated on ice for 1.5 to 2.5 hours, and infused intravenously 30-40 minutes after irradiation of the animal (after first having been filtered through a double layer of gauze). For neutraliz-
ing the heparin the dogs were injected intravenously with protamine sulfate (1 mg per 1 mg heparin) at the termination of the bone marrow infusion.

Bone marrow allografts were obtained from donors injected with 15–20 thousand units of heparin and electrocuted ten minutes later. The bone marrow was pressed out of the vertebra and heads of the long bones; it was placed in 400 ml of Hanks’ solution and centrifuged at 800–1000 r.p.m. for 5 minutes. After the fat had been removed the marrow cells were suspended in 500 ml of Hanks’ solution, filtered through a double layer of gauze and infused intravenously, 250 ml per animal (3.9 – 15 x 10^9 nucleated cells).

The erythrosin test showed the viability of the bone marrow cells obtained by aspiration to be 95 to 98 per cent; for the cells obtained by pressure it was 90 to 95 per cent.

Chimerism in cases of allogeneic bone marrow transplants was established by the presence of sex chromatin (the donors were females, the recipients males). The number of cells with characteristic drumstick processes of nuclear chromatin per 500 segmented neutrophils were counted. They constituted 4–6 per cent in the blood of the healthy females and 0–0.2 per cent in the males. Although this method is unsuitable for making a quantitative evaluation of chimerism, it may, however, be employed for determining the presence of donor granulocytes in the recipient organism. The animals were observed for periods of 5 months to 3 years and 2 months.

RESULTS

The control dogs (not given bone marrow transplants) exposed to the indicated doses of radiation always expired within the first two weeks following irradiation, displaying signs of an acute hemorrhagic syndrome and complete hematopoietic aplasia.

The dogs that had been subjected to bone marrow transplantation developed severe cases of radiation sickness; at the height of the disease they took no food, were very inert and lost much weight; however, in distinction from the controls, no visible hemorrhagic symptoms were observed in them. It is noteworthy that the clinical manifestations of acute radiation lesions in the dogs that had been given autologous bone marrow grafts were less pronounced; the appetites and activity of these animals were restored sooner, and their subsequent behavior was no different from that of normal unexposed dogs. Considerable loss of hair, to the extent of bald patches, was noted in all the exposed animals 1.5 to 2 months following irradiation. At remote dates the only objective sign that remained of the radiation lesion was the presence of focal patches of grey hair. The general condition of these animals was quite satisfactory: they were active and had good appetites; one of the females brought forth two litters of healthy pups.

Figure 1 presents data on changes in hematopoiesis during the period of active regeneration. It will be seen that the inhibition of blood formation in the dogs given autografts was not as extensive nor as protracted as with allografts. At the height of the disease, hypoplasia was approximately the same in both groups of animals; however, even at early stages (2 weeks) following exposure bone marrow punctates showed undifferentiated cells, immature cells of the myeloblastic series, and normoblastic elements. Neither leukopenia nor, in particular, thrombocytopenia were as advanced as in the allografted dogs, and the red blood values (Hb, erythrocyte count) were only slightly diminished. Bone marrow autografts were conducive to earlier and more active
regeneration of the myelokaryocyte count and of the absolute number of immature granulopoietic elements and the erythrocytic series. These values attained subnormal level by the fortieth day, while with allogeneic transplants such levels were attained no earlier than on the seventieth day following exposure. Similar rates of recovery were observed in the elements of the peripheral blood. The increase in the leukocyte and thrombocyte counts commenced at earlier dates and the process was much faster. In the animals that had received autografts the leukocyte and thrombocyte counts already attained subnormal levels 5 to 7 weeks following irradiation, in the allografted animals recovery took 3 to 4 weeks longer. Particularly pronounced differences were observed in the red blood values (Hb, erythrocyte count): the decrease was only 10–15 per cent in the case of autografts, as compared to the 50 per cent observed when allogeneic hematopoietic cells were introduced. The reticulocytic crisis preceding restoration of erythropoiesis was less marked
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in cases of autotransplantation than in allotransplantation, the maximums were reached on the twentieth and forty-eighth days respectively.

Lymphopoietic depression was of a similar intensity in both groups, and no differences were observed in the rates of recovery. The transient peak appearing in the lymphocyte curve on the eighth–tenth day following irradiation in dogs treated with allogeneic bone marrow is due to a partial transformation of the introduced hematopoietic cells into lymphocytes; this was discussed in the afore mentioned works.

Beginning with the tenth or twelfth day granulocytes containing sex chromatin were regularly found in the blood of the animals that had been given allografts. They constituted 0.5 to 2 per cent of the total cells count of segmented granulocytes. 28–35 days subsequent to transplantation granulocytes with drumstick nuclei had vanished from the blood of the recipients, and were not found any more during the entire period of observation. It may therefore be assumed that states of chimerism continued for only one month in our experiments, after which reversion occurred. The evolvement of complete radiochimeric dogs has been reported, in them the donor type of blood formation persisted for a protracted period of time (3–4 years). It would seem that the rapid reversion in our experiments was conditioned by an insufficient uniformity of x-ray radiation, the penetrating capacities of which are much lower than those of the gamma-emissions employed by the cited authors.

The period of active regeneration terminates within 2.5 months after irradiation in dogs given autologous and allogeneic transplants of bone marrow. Subsequently the character of blood formation was identical in both groups of animals (Fig. 2). For a protracted length of time hematopoiesis was unstable, periods of increase and decrease of all hematopoietic values alternating. They might be subnormal at the end of the regenerative period, and 2–3 months later pronounced hypoplasia would develop in the same animal. Such an instability continued for a long time. Thus, 4–5 months following irradiation bone marrow aspirates obtained from 4 dogs contained all the cellular elements; total cellularity reached 60–100 per cent of the initial values, while 5–8 months after exposure the myelokaryocyte count fell to 10–30 per cent of the initial values in the same animals. The changes in the immature white and red elements per 1 mm$^3$ of bone marrow were also marked by considerable instability. Restoration of granulopoiesis occurred gradually, and the content of immature elements in this series fluctuated considerably. Although the erythroblastic elements did normalize sooner, still no stable normalization of this hematopoietic line was observed either. The instability of blood formation was also reflected in the pattern of the peripheral blood. Three to four months after transplantation of allogeneic bone marrow, and somewhat earlier in the case of autotransplants (2–2.5 months) a secondary diminution in the leukocyte count was observed in a number of dogs; this condition was extremely pronounced in some cases (from 6000–10,000 to 4000–6000 cells per 1 mm$^3$); only 6 months following exposure was a slight tendency to increase noted. The leukocyte count returned to its initial level in 3 dogs after 7–12 months had passed, but later it again diminished considerably. For the duration
of 1–1.5 years a markedly fluctuating lymphopenia was observed in all the animals. Thrombocyte formation recovery was quite rapid in all the dogs; it remained at initial levels during long terms of observation. Unstable thrombocyte counts were observed in only two of the dogs that had received bone marrow allografts; a normal level was established in dog No. 96 only in 3 years.

The erythrocyte counts and hemoglobin values were practically unchanged in the dogs that had received bone marrow autografts, while in the animals given allografts initial values were attained only 3–4 months after exposure. In one case the anemic condition persisted for 1.5 years, showing no signs of recovery.
Fig. 3.—Hematopoietic tissue of irradiated dogs. Hematoxylin and eosin. (a) Bone marrow—lacunar bone resorption; osteoblasts; immature cellular elements. Dog. 159; × 280. (b) Bone marrow—fields of fat cells and areas of hyperplasia of active bone marrow near trabeculae. Development of collagenous tissue around the bone trabeculae. Dog. 188; × 56. (c) Bone marrow—osteoid in areas of resorption of the bone trabeculae; proliferation of osteoblasts; numerous reticular and immature cells in the bone cavity. Dog. 96; × 280. (d) Bone marrow—diffuse hyperplasia, numerous non-differentiated and immature cells, mitoses. Dog 181; × 560. (e) lymph node—marked decrease of number of follicles; accumulation of plasma cells and leukocytes in medullar layer. Dog 159; × 80. (f) spleen—follicles with a small zone of mature lymphocytes. Consolidation of stroma. Dog 101; × 80.

Seven of the nine dogs were killed 5 to 38 months following irradiation, and the conditions of their hematopoietic tissues was studied.

Changes, characterized by a thinning of the trabeculae and the formation of lacunae, were observed in the marrows of the flat bones of all seven animals (Fig. 3a). Wide bands of collagenous tissue were frequently found surrounding the thinned-out trabeculae. The collagen occupied not only the sites of bone
resorption, but also the bone sinuses and the adjacent bone marrow (Fig. 3b). In many sites of the zones of bone resorption an intensive proliferation of osteoblasts was noted. These cells frequently filled out the lacunae and were revealed among the adjacent bone marrow cells (Fig. 3c). In isolated areas the inner and outer layers of the endosteum were comprised of osteoblastic chains. An increase in the number of fat cells and an uneven distribution of the hematopoietic cells was observed; the latter were aggregated predominately in the vicinities of the osseous trabeculae (Fig. 3b). Fat cells, as a rule, were seen in the centre of the bone marrow cavities. In almost all the experiments the bone marrow was of a polymorphous composition, although a relatively higher count of reticulocytic cells and immature granulopoietic elements was observed. Aggregations of nucleated forms of the erythropoietic series were definable (Fig. 3a, c). Hemosiderosis was noted frequently.

Consequently, hypoplasia was present in most of the dogs; however, dog No. 181 showed pronounced hyperplasia of the bone marrow (8 months following irradiation). The number of fat cells had diminished sharply, the bone marrow cavities were full of reticular cells arranged in places in the form of syncytia. Accumulations of nucleate forms of the erythropoietic series, megakaryocytes, were visible among the reticular cells, and mitotic figures were seen (Fig. 3d). However, this hyperplasia was not evident in the bone marrow punctuates or in the peripheral blood: the myelokaryocyte counts were very variable (95–10–14 thousand per 1 mm³), leukopenia was noted in the peripheral blood.

Microscopic investigations showed a sharp diminution of lymphatic tissue in the variously localized lymph nodes (retrosternal, peribronchial, mesenteric, and inguinal). There were very few unevenly distributed lymphoid follicles (Fig. 3e). Some lacked reproduction centres, in others the centres were quite wide, but the lymphocyte zone was very narrow; sometimes the follicular lymphatic tissue was represented by immature lymphoid cells. The cellular elements were diffusely distributed over extensive areas of the cortical layer; there were numerous reticulocytes, plasmacytes, and polymorphonuclear cells among the lymphocytes. Lymphoid tissue was absent in the medullary layer; the extensive cellular accumulations observed consisted chiefly of plasmacytes and a large number of leukocytes, including eosinophils; reticular cells and megakaryocytes were also seen. Distinct proliferation of the cells of the reticuloendothelium and a large number of erythro- and siderophages were observed in the wide sinuses of the mesenterial lymph nodes.

In the spleen, as in the lymph nodes, the number of follicles was reduced; the stroma was compact and an extensive growth of fibrous tissue was discernible (Fig. 3f). These follicles were either very small, or they possessed wide centres and a zone of immature lymphoid elements. Abundant accumulations of plasmacytes were visible in the sinusoidal lumens on the trabeculae. In the splenic pulp focal accumulations of reticular cells, leukocytes, and nucleated erythroid forms were frequently discerned (dog no. 101, 159, 161, and 188). Megakaryocytes in various stages of maturity were usually found in these areas. In the case of dog no. 96 a particularly large number of reticular cells
and immature granulopoietic forms were observed. In some of the experiments the sinusoidal lumens also contained numerous erythrofages.

The presence of a large number of granulopoietic and erythropoietic cells in various stages of maturity and of megakaryocytes indicates that the spleen and lymph nodes were sites of extramedullary hematopoiesis in some of the experimental animals.

In four cases (dog no. 96, 159, 161, and 188) other ectopic sites of hematopoiesis were discovered. In three animals hematopoietic foci were found
in the depth of the myocardium, and in the right ventricular muscle in the area of the papillary muscles; these foci mostly appeared as formed bone marrow with reticular stroma, fat cells, and cellular elements of all three lines (Fig. 4a, b, c). A large number of erythroblasts were visible (Fig. 4d). Deposits of hemosiderin appeared in the cytoplasm in the areas of ectopic blood formation (Fig. 4b). No osseous trabeculae were visible in the newly formed bone marrow that adhered closely to the endocardium only in a few places. A proliferation of hematopoietic cells in the intermuscular spaces had occurred in some areas. In dog no. 161 the hematopoietic tissue on the periphery of the focus of its formation was represented by rows of erythroid cells, prevalently erythroblasts; closer to the centre the cell population was polymorphous. In dog no. 188 hematopoietic sites were also discovered in the lumens of the pulmonary vessels.

Hematopoietic tissue was discovered in dog no. 96 both in the tissue of the right heart and in the lumen of one of the hepatic vessels (Fig. 4e, f). A large number of immature erythroid and granulocytic cells, as well as plasmaocytes, were observed in these sites of ectopic hematopoiesis.

**DISCUSSION**

Experimental data show that active regeneration of hematopoiesis terminates within 2.5 months in lethally irradiated dogs. Later, despite the satisfactory clinical condition of the animals, hematopoiesis was gravely impaired practically throughout the entire term of observation (up to three years). It might have been assumed that this late disturbance in blood formation was associated with the fact that stem cells were responsible in these cases for it, and since these cells had been seriously injured by the radiation they were incapable of complete realization of their hematopoietic potentialities. However, such an assumption does not seem very likely since in cases of autologous transplantation of bone marrow unexposed hematopoietic cells are evidently responsible for blood formation. It is true that the absence of any markers in cases of bone marrow autografts make it impossible to affirm definitely that the transplanted population of hematopoietic cells actively repopulating and proliferating in the period of active regeneration was subsequently not depleted or replaced by regenerating irradiated elements. An yet, such an assumption seems quite plausible if only for the reason that syngeneic chimeras have very little propensity for reversion. Moreover, it has been demonstrated recently that following the introduction of only 5000 syngeneic bone marrow cells into sublethally irradiated mice the recipient's hematopoietic cells are sooner or later completely replaced by nonirradiated cells of the donor. Consequently, even a very intensive and protracted proliferation does not deplete the small pool of stem cells received by the recipient. Therefore it seems more probable that the development of late hematopoietic lesions is caused by impairment of the structure of the hematopoietic tissue, notably the detected bone tissue lesion. Most illustrative in this respect are the data concerning the onset of a serious disturbance of hematopoiesis (subsequent to good initial regeneration) even in a very small irradiated area of a rat femur when the
prevailing mass of bone marrow is shielded; here active repopulation proceeds continuously, but despite this hematopoiesis is not restored to normal in the exposed area. Consequently, the mere presence of undamaged hematopoietic stem cells is insufficient for maintaining completely normal hematopoiesis. Characteristic features are uneven distribution of the hematopoietic cells and the presence of extensive areas of fat marrow. The cellular population is polymorphous in the foci of blood formation; however, a relative increase in the number of erythroid cells is noted, as well as a variously expressed growth of the immature granulopoietic cells and aggregations of reticular cells. The latter may be so pronounced (dog no. 181) that it resembles a diffuse proliferation of reticular cells accompanying leukemia, although the hematopoietic impairment is retained. This indirectly confirms that the reticular cells are not hematopoietic stem cells, an opinion that has of late been receiving ever-increasing acknowledgment. Another characteristic feature is the presence of multiple sites of ectopic hematopoiesis, notably in the lymph nodes and the spleen. Hematopoietic sites were also discovered in the lungs, right heart, and liver of a number of the experimental animals. In these sites the hematopoietic tissue was usually represented by stroma with granulocytic and erythroid cells, megakaryocytes, and fat cells in its loops. Reticular cells and even accumulations of hemosiderin were also observed. The cellular population in the ectopic hematopoietic tissue was very similar to that of the bone marrow.

The smallness of the sites of ectopic blood formation and their weak association with the vascular wall permits the assumption of the transiency of such foci that evidently continuously appear and vanish during a considerable length of time following irradiation. An autochthonous formation of foci of heterotopic hematopoiesis on the basis of local cambial elements is highly improbable. It is more likely that after irradiation large numbers of stem cells continuously emerge into the blood stream. They lodge in various areas of the vascular system, forming hematopoietic sites that are sooner or later depleted. It is possible that acute impairment of bone structure accompanied by resorption and incomplete regeneration of the osseous trabeculae is one of the conditions necessary for breaking down the barrier that normally controls the emergence of bone marrow cells into the blood circulation.

It is notable that the formation of ectopic foci of hematopoiesis was not attended by bone formation. No normal osteogenesis took place, as has already been pointed out, in the normal areas of hematopoiesis, even in cases of autologous bone marrow transplantation. Consequently, the presence of undamaged hematopoietic stem cells is insufficient for the induction of normal osteogenesis under the given conditions.

As will be seen from the above data, regeneration of lymphopoiesis in dogs protected by autologous bone marrow took place at the same time as it did in cases of allotransplantation of bone marrow. Consequently, under these conditions the introduced autologous hematopoietic cells differentiated in all three lines of hematopoiesis, while differentiation in the lymphoid direction was either absent, or very slow and difficult. This was confirmed histologically by
Scalp Diseases
Hematopoiesis


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