Defective Capacity of Bone Marrow From Nude Mice to Restore Lethally Irradiated Recipients

By Dov Zipori and Nathan Trainin

The proliferative capacity of bone marrow cells from thymus-deprived nude mice was investigated in lethally irradiated recipients. Although the colony-forming capacity of these cells was found to be similar to that of normal littermates, a reduction in the number of nucleated cells was observed in the bone marrow of nude mice. Moreover when the radioprotective effect of such cells was studied, it was found that $5 \times 10^5$ or $2 \times 10^6$ bone marrow cells from nude mice were less effective in restoring hemopoiesis and establishing permanent chimerism than similar amounts of bone marrow cells of normal controls. In addition, irradiated animals surviving after injection of bone marrow cells from nude mice were found to have lower immune responses to SRBC than normal chimeras. The possibility that mortality of irradiated recipients injected with bone marrow of nude mice is due to the presence of a latent infective agent or of some inhibitory factor of hematopoiesis in the bone marrow of such nude mice is shown to be improbable. Alternatively it is suggested that nude mice suffer from an intrinsic defect in the proliferative capacity of their bone marrow colony-forming cells (CFUs).

Hairless mice, homozygous for the nude mutation, have been shown to suffer from defective development of the thymus. Studies performed in the last few years revealed that nude mice lack thymocytes and thymus-derived cells. Consequently, their immune reactivity to certain antigens and their capacity to reject allogeneic skin grafts are reduced. Normal function of the thymus is dependent upon a pool of precursor cells originating from yolk sac and fetal liver in embryonic life and from the bone marrow in adult life. It has been suggested that absence of thymocytes in nude mice is a result of a defect in the thymic epithelium, rather than deficiency in precursors of thymocytes.

From the point of view of immunological reactivity the “nude syndrome” is similar to the wasting syndrome which usually follows neonatal thymectomy in mice. It has been shown in our laboratory that thymus-deprived mice, thymectomized at 3 days of age, show a reduction in the colony forming capacity of their bone marrow cells. Moreover, histological examination of the spleen nodules showed that the distribution of colony types obtained following inoculation of normal bone marrow was different from that obtained with bone marrow from thymectomized donors, the latter producing a higher ratio of erythroid to granuloid colonies. The reduction in the colony forming capacity of bone marrow of thymectomized mice could, therefore, be due to changes in the intrinsic nature of the colony forming unit (CFU) population, rather than to a simple decrease in the number of CFUs. This assumption is
further substantiated by the fact that the protective capacity of bone marrow of thymectomized animals, upon injection into lethally irradiated mice, is impaired. The above experiments suggest that the absence of the thymus following neonatal thymectomy causes serious changes in the control of hematopoiesis occurring in the bone marrow. Such changes could not be demonstrated in the spleens of thymectomized mice, and we have postulated that stem cells present in the bone marrow only are under the control of the thymus. In light of the above observations, and since the thymus functions abnormally even in fetal life in the nude mice, we expected to find striking changes in the bone marrow cell behavior of these mice. This communication presents our studies on the capacity of the bone marrow of nude mice to protect lethally irradiated animals and to establish permanent chimerism.

MATERIALS AND METHODS

Mice

Male mice, heterozygous for the "nude" mutation, kindly donated by Prof. D. W. van Bekkum, TNO, Radiobiological Institute, Holland, were mated with female C3H/eh mice. The F1 offspring were then mated randomly. In the experiments described below F1 hairless mice of both sexes and their normal littermates were used as experimental animals and controls, respectively. Inbred C3H/eh mice, originally obtained from Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, were bred at the Weizmann Institute by sibling mating. Female animals, at the age of 80-100 days, were irradiated and used as recipients of bone marrow cells. The irradiated animals were housed in metal cages in airconditioned rooms kept at 24-26°C, and fed Purina Laboratory Chow pellets and tap water ad libitum. Each cage contained no more than two mice.

Irradiation

Mice were exposed to a single dose of 900 R from a Gammabeam, 150-A, 60Co source, produced by Atomic Energy of Canada, with F.S.D. of 75 cm and 65 R/min dose rate.

Preparation of Bone Marrow Cell Suspensions

Bone marrow was obtained from the tibiae and femora of each mouse. In some experiments bone marrow cells from two to four donors of the same sex were pooled. Donor mice were 10-60 days of age, according to the experiment. In each experiment, experimental and control mice were of the same age. A 26-gauge needle mounted on a 1.0-ml syringe containing Tyrode’s solution (pH 7.4) was inserted into one end of the bone, and the marrow was discharged by forcing the solution through the marrow cavity. Nucleated cells were counted in a hemocytometer.

CFU Assay

Details of the procedure have been previously described. Bone marrow suspensions were adjusted by dilution with Tyrode’s solution to a concentration of 3 x 10⁶ cells per 0.5 ml. The suspensions were injected into the caudal veins of a group of 15 irradiated recipients. Each mouse received 0.5 ml of the solution.

Radioprotection Assay

Bone marrow suspensions, prepared as indicated before, containing either 5 x 10⁵ or 2 x 10⁶ cells in 0.5 ml volume, were injected intravenously into 30 irradiated recipients. Mortality was checked daily from the day of irradiation. Dead mice were autopsied, and samples of their tissues were fixed and examined histologically.
DEFECTIVE CAPACITY OF BONE MARROW FROM MICE

Fig. 1. Number of nucleated cells in the bone marrow of nude mice (•) and of normal littermates (○). Every circle is an average of two-five animals.

Antibody Responses to Sheep Red Blood Cells (SRBC)

Mice were injected intraperitoneally with 0.5 ml of a 10% SRBC suspension in saline. Five days later the mice were bled and their spleens were removed. Hemolsin and hemagglutinin titers were determined by standard methods described elsewhere.14 The plaque-forming cell assay was performed by the classical technique of Jerne.15 Statistical analysis of data was performed by the Student's t test.

RESULTS

Reduced Number of Nucleated Cells in the Bone Marrow of Nude Mice

Changes in the total number of nucleated bone marrow cells from both hind limbs of individual nude mice and their littermate controls between 10 and 60 days of age are shown in Fig. 1. During this period the total number of bone marrow cells in nude mice decreased as compared to that in normal littersmates. As in other strains of mice,16 we also observed a constant increase in the number of bone marrow cells during the first month of life. At the age of 30-40 days the total number of nucleated cells in the bones examined reached a plateau of about 40 × 10^6 cells in normal mice, and about 30 × 10^6 cells in nude mice. However, it should be emphasized that only few nude mice kept under conventional conditions survive for up to 60 days.1 The number of nu-

Table 1. Relative and Absolute Number of CFUs in the Bone Marrow of Nude Mice and Their Normal Littermates

<table>
<thead>
<tr>
<th>Age of Donor (Days)</th>
<th>Relative Number of CFUs (CFUs per 3 × 10^6 bone marrow cells)</th>
<th>Absolute Number of CFUs (CFUs in Four Bones)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nude</td>
<td>Normal</td>
</tr>
<tr>
<td>10</td>
<td>1.0</td>
<td>3.0</td>
</tr>
<tr>
<td>10</td>
<td>4.1</td>
<td>5.1</td>
</tr>
<tr>
<td>15*</td>
<td>3.6</td>
<td>3.0</td>
</tr>
<tr>
<td>19</td>
<td>2.1</td>
<td>2.3</td>
</tr>
<tr>
<td>53</td>
<td>4.6</td>
<td>6.5</td>
</tr>
<tr>
<td>73t</td>
<td>4.0</td>
<td>5.9</td>
</tr>
</tbody>
</table>

* Pooled bone marrow of two mice.
† Pooled bone marrow of three mice.
cleated cells in nude mice of this age represents, therefore, the bone marrow content of a highly selected population.

**Determination of the Colony Forming Capacity of Bone Marrow of Nude Mice**

Table 1 shows the results obtained in different experiments performed with bone marrow donors of five different ages. Bone marrow cells (3 x 10⁴) of nude mice and normal littermates were injected intravenously into separate groups of 15 irradiated recipients each. The spleen colonies were counted 8 days after inoculation. The relative number of CFUs, i.e., the number of CFUs per 3 x 10⁴ bone marrow cells, in the bone marrow of nude mice did not differ markedly from that of their normal littermates. The absolute number of CFUs in the four bones studied, calculated by means of the following formula,

$$\frac{\text{No. nucleated cells in BM}}{3 \times 10^4} \times \text{No. CFU per spleen},$$

decreased in nude mice, as expected from their reduced nucleated cell counts (Fig. 1).

**Defective Radiation Protection Capacity of the Bone Marrow of Nude Mice**

The radioprotective effect of bone marrow grafts depends mainly upon the number and intrinsic nature of the stem cells present in the graft.¹⁷,¹⁸ In addition, differentiated cells and noncellular factors participate, under certain conditions, in the mechanism of radioprotection.¹⁹ In the following experiments the radioprotective capacity of bone marrow of nude mice was investigated. Bone marrow cells (5 x 10⁴ or 2 x 10⁴) obtained from nude mice from 10 to 60 days of age, and equal numbers of cells from their normal littermates, were injected separately into groups of 30 irradiated recipients. Six separate experiments were performed, in which a marked decrease in the capacity of the bone marrow of nude mice to protect lethally irradiated mice could be shown. Figure 2 summarizes cumulative mortality curves obtained in one representative experiment. Neither 5 x 10⁴ nor 2 x 10⁴ bone marrow cells from 40-days-old nude mice were able to protect lethally irradiated mice. On the other hand, bone marrow cells from normal littermates were shown to be as effective as syngeneic C3H/eb bone marrow in their protective capacity, upon injection into irradiated mice. As seen in Fig. 2, 160 days after irradiation 100% and 90% mor-

![Cumulative mortality of C3H/eb mice lethally irradiated and injected with 5 x 10⁴ (o-o) or 2 x 10⁴ (---) bone marrow cells of nude mice, 5 x 10⁵ (---o) or 2 x 10⁵ (---e) bone marrow cells of normal littermates, and 2 x 10⁴ (---) bone marrow cells from normal C3H/eb mice. In each of these groups bone marrow cells from four donors were pooled. This figure is representative of six similar experiments all showing the same pattern.](chart.png)
tality occurred among the groups of mice previously injected with $5 \times 10^6$ and $2 \times 10^6$ bone marrow cells, respectively, from nude mice, as compared to 25% and 15%, mortality among mice injected with the same doses of bone marrow cells from normal littermates.

These results suggest that bone marrow cells of nude mice have an intrinsic defect in their capacity for self-renewal, which limits the period of protection the cells provide when administered to lethally irradiated mice. The fact that bone marrow from nude mice contains a normal relative number of CFUs (Table 1) while at the same time having a reduced radioprotective capacity (Fig. 2) may, however, raise alternative explanations. It can be argued that the bone marrow of nude mice carries some latent infection or inhibitory factor of cell proliferation which, upon administration to the recipient mouse, contributes to its death. To investigate this possibility, the capacity of bone marrow cells from nude mice per se, and in a mixture with bone marrow cells from normal mice, was tested. Bone marrow cells ($5 \times 10^6$) of normal and nude mice were injected separately into groups of 30 irradiated recipients, to serve as controls. Mice of the experimental group received a mixture of cells containing $5 \times 10^6$ bone marrow cells from normal donors and $5 \times 10^6$ bone marrow cells from nude donors. Three separate experiments were performed, and the results of one representative experiment are tabulated in Fig. 3. As can be seen, addition of bone marrow from nude mice to bone marrow cell suspensions of normal mice did not reduce the radioprotective capacity of the latter, but increased it slightly. Moreover, macroscopic examination of recipient mice immediately after death, and histological preparations of their tissues, failed to show any manifestation of an infective process which could have been the direct cause of their death.

Low Immune Response to SRBC of Radiation Chimeras Established by Bone Marrow Grafts From Nude Mice

Prolonged survival of radiation chimeras depends, among other factors, upon restoration of immune responsiveness.20 The following experiments were carried out in order to investigate the immunological reactivity of irradiated...
recipients restored by bone marrow from nude mice. Bone marrow cells ($2 \times 10^6$) from nude or normal mice were injected into lethally irradiated recipients. One hundred and ten days after this treatment, survivors of both groups were injected with SRBC. Since it was found that some recipients of bone marrow from nude mice had, at that time, a decreased body weight, immune responses to SRBC were checked only in the recipients whose body weights were similar to those of the controls. This prevented the inclusion of animals with immune reactions possibly impaired by unrelated reasons, such as runting or secondary infections.

As can be seen in Fig. 4 and Table 2, 5 days after immunization with SRBC the titers of hemagglutinin and hemolysin in the sera and the number of PFC in the spleens of recipients of bone marrow from nude mice were lower than those of the controls.

DISCUSSION

The observations presented here, together with previous results obtained with neonatally thymectomized animals, indicate that the absence of normal thymic tissue alters the pattern of hematopoietic control exerted by the marrow. In the case of neonatal thymectomy, we have shown that in the absence of the thymus, a decrease in the colony forming capacity of bone marrow cells develops which is accompanied by a reduced capacity of these bone marrow cells to protect lethally irradiated animals. A similar phenomenon was found in the present case when nude mice were used as donors. Bone marrow cells of these

<table>
<thead>
<tr>
<th>Donors</th>
<th>Recipients</th>
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<tbody>
<tr>
<td>Nude</td>
<td>0, 30, 103, 119, 151 (average 82)</td>
</tr>
<tr>
<td>Normal</td>
<td>112, 141, 298, 308, 353 (average 242)</td>
</tr>
</tbody>
</table>

*PFC determined 5 days after injection of SRBC.
† Each number represents PFC/10$^6$ spleen cells.
$\not p < 0.05$
mice were unable to restore lethally irradiated female C3H/eb recipients. The mortality of the recipients in the present experiments cannot be explained on the basis of genetic incompatibility between the bone marrow cells of the nude donors and of the C3H/eb recipients. Bone marrow cells from normal littermates of nude mice and bone marrow from normal syngeneic C3H/eb mice had, indeed, an equal protective capacity upon injection into C3H/eb recipients. Alternatively it may be argued that the death of animals injected with bone marrow from nude mice resulted from the presence of contaminating microorganisms, or from some inhibitory factor of hematopoiesis contained in the bone marrow of these mice, which would be transferred to the recipients. However, addition of \( 5 \times 10^5 \) bone marrow cells from nude mice to an equal amount of normal bone marrow cells did not increase the mortality of the recipients, as compared to that of irradiated recipients of normal bone marrow only. It should be emphasized that \( 5 \times 10^6 \) normal bone marrow cells are not capable of restoring 100%, of the irradiated recipients. Addition of any inhibitory agent would, therefore, be expected to alter strongly the radioprotective effect of such cell suspensions. On the basis of these results we find it unlikely that the mortality of mice injected with bone marrow from nude mice is attributable to the presence of any defined contaminant or inhibitory factor. We suggest, therefore, that nude mice suffer from a defect in the proliferative capacity of their bone marrow. The relative number of CFUs in the bone marrow of nude mice is similar to that of normal controls, but the total number of CFUs is lower. Moreover, this total remains nearly unchanged during the first 60 days of life in nude mice, in contrast to a gradual increase in the total number of CFUs in normal controls. In light of all these results, it appears that the self-renewal capacity of CFUs in nude mice is impaired. Further studies are being carried out in our laboratory to investigate this possibility.

Although in our experiments permanent chimerism was not generally observed when bone marrow from nude mice was injected into lethally irradiated recipients, a few mice injected with \( 2 \times 10^6 \) bone marrow cells from these donors survived until 4-5 mo after irradiation. These survivors behaved differently from recipients of normal bone marrow as regards their immunological reactivity to SRBC and were characterized by a lower level of circulating hemolysins and hemagglutinins, and by a lower capacity of PFC formation in their spleens after stimulation by SRBC. Since the irradiated recipients possessed their own thymus which could eventually induce immunological maturation of cells originating in the bone marrow of the donor animals, the present findings suggest that the bone marrow of nude mice is deprived of a defined thymus-dependent population.

These observations would, therefore, imply that normal bone marrow contains a population of "prethymic" stem cells already committed to a pathway of differentiation controlled by the thymus. It has been postulated that normal proliferation of cells in the lymphohemopoietic tissue is regulated by feedback loops interconnecting the various organs of the system and determining the rate of proliferation and size of these organs. Such a model would imply that removal of the thymus will affect not only the thymus-derived cell population, but also the proliferation of cells in the bone marrow, which is the source of lym-
phoid precursors. As demonstrated by the present results, absence of the thymus induces alterations in the CFU population of the bone marrow. This suggests the presence of a feedback mechanism between the thymus and the bone marrow, interconnecting these two related organs. The existence of such mechanism has not been entirely demonstrated, but the possibility that humoral factors secreted by the thymus exert a regulatory function on bone marrow activity has been suggested.12,22

ACKNOWLEDGMENT

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REFERENCES

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