Development of Hematopoietic Spleen Colonies in Nonirradiated Genetically Normal Mice

By Y. Kitamura, M. Tamai, Y. Miyano, and M. Shimada

The question as to whether prior irradiation or injection of cytotoxic drugs is essential for the development of spleen colonies was examined in genetically normal mice. Mixtures of lymph node and bone marrow cells from C57BL mice were injected into (C57BL x CBA-T6T6) F1 hybrid mice without pretreatment. Hematopoietic nodules were observed in the CFU-S spleens of F1 hybrid mice killed 1-8 days after injection. The average number of nodules increased linearly with increased numbers of injected bone marrow cells. Histologic examination showed that erythroid cell colonies predominated over granulocytic cell colonies. These results suggest that any kind of treatment that causes the depletion of CFU-S in the spleen of hosts would provide a suitable environment for the production of colonies by transplanted CFU-S.

Since the spleen colony method was first described by Till and McCulloch, it has been one of the most useful tools for the investigation of hematopoietic progenitor cells. However, the conditions that permit the formation of colonies in the spleen have not been studied thoroughly. In most experiments, spleen colonies were produced in lethally irradiated mice. Mice injected with cytotoxic drugs and W/WV anemic mice, whose spleen colony-forming units (CFU-S) are genetically defective, were also used as hosts for colony formation. In all these cases, there were actually no effective CFU-S in the spleens of the hosts when the cell suspensions containing CFU-S were injected. It is therefore possible that the absence of CFU-S in the spleen might be a prerequisite for the development of colonies in the spleen. In order to examine this possibility, we attempted the production of hematopoietic colonies in the spleens of genetically normal mice without irradiation or injection of cytotoxic drugs. Allogeneic lymph node cells were used as a means for eradication of CFU-S of the hosts, according to Davis et al.

MATERIALS AND METHODS

MICE

Male BT6Fi (C57BL x CBA-T6T6) hybrid mice and male C57BL mice were used at 2-3 mo of age. C57BL, CBA-T6T6, and BT6F1 mice were raised in our laboratory.

CELL SUSPENSIONS

Cells were suspended in Eagle's medium as previously described. Cells in 0.2-0.8 ml medium were injected via a lateral tail vein. When more than 50 x 10^6 cells were used, 50 lU of sodium hepanin was injected intraperitoneally (i.p.) 20 min before the cell injection.

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Irradiation

Irradiation was undertaken with a Toshiba x-ray machine operated at 180 kV and 20 mA (target distance, 72 cm; 1-mm aluminum filter; 61 rad/min). BT6F₁ mice received 850 rads and C57BL mice 790 rads. The cells were injected into the mice within 3 hr after irradiation.

Colony Formation in Nonirradiated Mice

Nonirradiated BT6F₁ mice were injected intravenously with cells of immunized C57BL mice that had received spleen and lymph node cells of CBA-T6T6 mice (20 × 10⁶) i.p. twice at 7-day intervals, and were killed 7 days after the last injection. The parental bone marrow cells (BMC) were injected simultaneously with the parental lymph node cells (LNC), or the BMC were injected 3, 6, or 9 days after the injection of the LNC. In all cases the F₁ recipients were killed on the 18th day after the injection of the LNC (Fig. 1). Control BT6F₁ mice that received syngeneic LNC and BMC were also killed on the 18th day after cell injection. The spleen was removed and fixed in Bouin’s solution. Colonies were counted with a dissection microscope (×7). In one experiment, the spleen was cut at 50-μm intervals; sections were stained with hematoxylin and eosin. Identification and quantification of colonies were performed according to the criteria of Curry and Trentin.⁷

Number and Origin of CFU-S

The method of Till and McCulloch¹ was used to assay the number of CFU-S in the spleen. Spleens were removed from four nonirradiated BT6F₁ mice on different days after the injection of parental or syngeneic cells; the cells of these four spleens were suspended in 10 ml Eagle’s medium. A known proportion was injected into 12–20 irradiated mice. The mice were killed on the 8th day after irradiation and cell injection. The macroscopic colonies were counted with the same method used in the case of nonirradiated mice. In one experiment, irradiated C57BL mice that had been immunized with five injections of CBA-T6T6 cells were used as the recipients to determine the origin of CFU-S.

Chromosome Analysis and Mitotic Index

Mice were injected with colchicine i.p. (0.04 mg/mouse) 2 hr before killing. The spleens were removed and cut transversely into two equal parts. Chromosome preparations were obtained according to the method of Kunita et al.⁸ from one of the parts. Subserial sections of the other part were made after fixation in Bouin’s solution. The mitotic index was calculated according to the method of Leblond and Stevens.⁹ From every 10 spleens, 3 erythroid colonies, 3 lymph follicles, and 3 areas of red pulp without colonies were examined. In each part, more than 1000 nuclei were counted under oil (×1000) immersion.

RESULTS

Development of Spleen Colonies

Nonirradiated BT6F₁ hybrid mice were injected with 75 × 10⁶ LNC of C57BL mice. When the F₁ hybrid mice were killed on the 18th day after the
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Table 1. Dose and Origin of Lymph Node Cells Necessary for the Production of Spleen Colonies in Nonirradiated BT6F1 Mice

<table>
<thead>
<tr>
<th>Donor Cell</th>
<th>Cell Dose* (x 10^6)</th>
<th>No. of Mice</th>
<th>Colony Development (%)t</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LNC</td>
<td>BMC</td>
<td></td>
</tr>
<tr>
<td>C57BL</td>
<td>0</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>C57BL</td>
<td>25</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>C57BL</td>
<td>50</td>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>C57BL</td>
<td>75</td>
<td>1</td>
<td>39</td>
</tr>
<tr>
<td>BT6F1</td>
<td>75</td>
<td>1</td>
<td>21</td>
</tr>
</tbody>
</table>

LNC, lymph node cells; BMC, bone marrow cells.

Cent of BT6F1 mice in which spleen colonies were grossly detected on the 18th day after cell injection.

cell injection, the surface of the spleen was smooth and no nodules could be seen. Histologic examination revealed scarcely any hematopoietic foci. The injection of the parental BMC (10⁶ or 20 x 10⁶) alone produced no nodules either (Table 1). In contrast, when the mixture of parental LNC (75 x 10⁶) and BMC (10⁶) was injected into nonirradiated BT6F1 mice, hematopoietic nodules were observed in the spleen 16-22 days after the injection (Fig. 2).

In the next experiment, BT6F1 mice were killed on the 18th day after the injection of a varying number of parental LNC and a fixed number of parental BMC (10⁶). Although the injection of 75 x 10⁶ LNC and the BMC produced nodules in all spleens of the BT6F1 mice examined, nodules were observed only in 25% of the F1 mice that had received 25 x 10⁶ LNC and the BMC (Table 1). With regard to the donor of the cells, the injection of LNC (75 x 10⁶) and BMC (10⁶) of the BT6F1 mice did not produce any colonies in the spleen of the syngeneic mice (Table 1).

Time Between Injection of LNC and BMC

Parental BMC were injected into the nonirradiated BT6F1 mice 3, 6, or 9 days after the injection of parental LNC (75 x 10⁶), and the mice were killed 15, 12, or 9 days, respectively, after the injection of the BMC (Fig. 1). In these

Fig. 2. Hematopoietic nodules in the spleen of nonirradiated BT6F1 mouse that had been injected with a mixture of lymph node and bone marrow cells of C57BL mice 18 days before.
The origin of hematopoietic cells in the spleen was examined in two experiments. First, the number and the origin of the CFU-S were determined on different days after the injection of the syngeneic or the parental LNC (75 x 106) into nonirradiated BT6F1 mice. Although the injection of syngeneic LNC did not reduce the number of CFU-S in the spleen, the injection of parental LNC eradicated the CFU-S from the spleen by the fifth day (Table 2). On the other hand, the spleens of the BT6F1 mice injected with the mixture of parental LNC (75 x 106) and BMC (4 x 106) 18 days before contained a considerable number of CFU-S. These CFU-S were able to develop into colonies in the irradiated C57BL mice that had been immunized against CBA-T6T6 cells (Table 2). As the injection of 3.4 x 106 spleen cells of the normal BT6F1 mouse
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Table 2. Number of CFU-S in the Spleens of Nonirradiated BT6F1 Mice on Different Days After Injection of Parental or Syngeneic Cells

<table>
<thead>
<tr>
<th>Donor Cell</th>
<th>Days After Injection of Cells</th>
<th>Cell Dose(^*) ((\times 10^6))</th>
<th>No. of Mice</th>
<th>No. of CFU-S per Spleen (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal†</td>
<td>—</td>
<td>0</td>
<td>0</td>
<td>291</td>
</tr>
<tr>
<td>C57BL</td>
<td>3</td>
<td>75</td>
<td>0</td>
<td>*151</td>
</tr>
<tr>
<td>C57BL</td>
<td>4</td>
<td>75</td>
<td>0</td>
<td>17†</td>
</tr>
<tr>
<td>C57BL</td>
<td>5</td>
<td>75</td>
<td>0</td>
<td>7†</td>
</tr>
<tr>
<td>C57BL</td>
<td>9</td>
<td>75</td>
<td>0</td>
<td>7†</td>
</tr>
<tr>
<td>C57BL</td>
<td>18</td>
<td>75</td>
<td>0</td>
<td>13†</td>
</tr>
<tr>
<td>BT6F1</td>
<td>18</td>
<td>75</td>
<td>4</td>
<td>19§</td>
</tr>
<tr>
<td>BT6F1</td>
<td>18</td>
<td>75</td>
<td>0</td>
<td>11†</td>
</tr>
</tbody>
</table>

* LNC, lymph node cells; BMC, bone marrow cells.
† Spleens were taken from normal BT6F mice; pooled data from three experiments.
‡ Normal C57BL mice were used as the recipients after irradiation.
§ Immunized C57BL mice were used as the recipients after irradiation.

(13.8 CFU-S when assayed in irradiated BT6F mice) did not produce any colonies in the immunized C57BL mice, the CFU-S assayed in the immunized C57BL mice could be inferred to be of C57BL mouse origin.

In the next experiment, chromosome analyses were carried out using the spleens of the BT6F mice that had been injected with LNC (75 \(\times 10^6\)) and BMC (2 \(\times 10^6\)) of C57BL mice 18 days before. Almost all cells in mitosis were of donor origin. The mitotic indices of the erythroid cell colonies were much higher than those of other parts of the spleen (i.e., lymph follicles and red pulp without colonies). As the injection of LNC alone scarcely produced colonies, the cells dividing in the erythroid cell colonies seemed to come from the BMC of the C57BL mice.

Differentiation Pattern of Colonies

The spleens of BT6F mice that had been injected with parental LNC and BMC were examined histologically. Erythroid cell colonies were predominant both in the spleens of the F1 hybrid mice receiving the parental LNC and BMC simultaneously and in the spleens of the F1 hybrid mice receiving the parental BMC 9 days after the injection of the parental LNC (Table 3).

Table 3. Differentiation Pattern of Colonies in the Spleens of Nonirradiated BT6F1 Mice on the 18th Day After the Injection of Parental Lymph Node Cells

| Day of BMC\(^*\) | Cell Dose (\(\times 10^6\)) | No. of Mice | Mean No. of Colonies of Type\(|\) | E-G Colony Ratio\(|\) |
|------------------|-----------------------------|-------------|-------------------------------|----------------------|
| Injection        | LNC†                        | BMC         | Ery, Gra, Meg, Un, Mix, Total  | Eryoid to granulocytic |
| —                | 75                          | 0           | 12                            | 0.3                  | 0.4                  | 3.0          |
| 0                | 75                          | 1           | 8                             | 24.1                 | 29.9                 | 6.7          |
| 9                | 75                          | 0.05        | 10                            | 11.5                 | 17.6                 | 2.7          |

* BMC, bone marrow cells.
† LNC, lymph node cells.
‡ Ery, erythroid; Gra, granulocytic; Meg, megakaryocytic; Un, undifferentiated; Mix, mixed.
§ Erythroid to granulocytic ratio.
Hematopoietic cell colonies developed in the spleens of nonirradiated BT6F1 mice that had been injected with lymph node cells (LNC) and bone marrow cells (BMC) of C57BL mice. As the injection of parental LNC alone eradicated the CFU-S from the spleens of the F1 hybrid hosts and scarcely made any colonies, and as the CFU-S and the dividing hematopoietic cells were shown to be of C57BL origin, the colonies in the nonirradiated F1 mice were derived from the BMC of the C57BL mice. On the other hand, the injection of syngeneic LNC did not reduce the number of CFU-S in the spleens of the BT6F1 mice, and the simultaneous injection of syngeneic LNC with BMC did not produce any colonies. These results suggest that any treatment that causes the depletion of CFU-S would lead to a suitable environment for colony formation by CFU-S.

Spleen colonies in the nonirradiated mice could not be detected before the 16th day after cell injection, in spite of the fact that spleen colonies were grossly detected on the 6th day after cell injection into irradiated mice. As the size of the colonies found on the 18th day after the injection of parental LNC and BMC was nearly equal to the size of colonies in the F1 hybrid mice that had been injected with parental BMC 3, 6, or 9 days after receiving parental LNC and were killed on the 18th day after the LNC injection, the CFU-S of parental origin did not seem to start proliferation for the first 9 days even if the CFU-S were injected with LNC at the same time.

The injection of parental BMC 6 or 9 days after the LNC injection produced about 10 times more colonies than the simultaneous injection of BMC with LNC. As the BT6F1 hybrid mice have been reported to resist the growth of CFU-S from C57BL mice, and as Cudkowicz and Stimpfling and Goodman and Wheeler have reported that the graft-versus-host (GVH) reaction preceding the injection of the CFU-S abrogates the hybrid resistance, the increased efficiency of the BMC injection may be explained by the abolition of the hybrid resistance during the first 6 days.

Erythroid cell colonies were predominant in the spleens of nonirradiated BT6F1 mice receiving parental LNC and BMC. This differentiation pattern was usually seen in the spleens of the irradiated mice receiving the syngeneic CFU-S. On the other hand, Kitamura et al. and Basford and Goodman reported that granulocytic cell colonies were more abundant than erythroid cell colonies in the spleens of irradiated F1 hybrid mice that had been injected with parental LNC and BMC. However, this suppressing effect of the GVH reaction on erythropoiesis was dependent both on the irradiation dose of the recipients and on the injection dose of the parental LNC when the irradiated F1 hybrid mice were used as the recipients of the parental cells. With the smaller irradiation dose, more LNC were necessary. Thus, it is possible that a higher dose of parental LNC changed the differentiation pattern of the parental CFU-S in the nonirradiated F1 hybrid mice.

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REFERENCES

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