Hematologic Changes During Spleen Colony Development in Nonirradiated Mice

By M. Tamai and Y. Kitamura

The mechanism of spleen colony formation in nonirradiated mice was investigated. When the spleen cells of C57BL mice immunized against CBA-T6T6 mice were injected into the nonirradiated BT6F1 (C57BL x CBA-T6T6) hybrid mice, the number of hematopoietic stem cells (CFU-S) of C57BL mouse origin that settled in the spleen of the BT6F1 mice continued to decrease in the first 9 days and then started to increase, with a doubling time of about 36 hr. Colonies were detected on the surface of the spleen 16–22 days after the cell injection. The slower appearance of spleen colonies in nonirradiated mice (comparing with 6–10 days in the irradiated animals) appears to be due to retarded start of differentiation and to the prolonged doubling time of CFU-S in nonirradiated mice.

Many investigations have been carried out concerning the proliferation and differentiation of hematopoietic stem cells (CFU-S) in irradiated hosts. When the cell suspension containing CFU-S is injected into lethally irradiated mice, the CFU-S settles in hematopoietic tissues, replicates itself, and differentiates into functioning descendants. A macroscopic colony formed in the spleens of irradiated mice is the result of proliferation and differentiation of a transplanted CFU-S.

Recently, we described the development of hematopoietic colonies in nonirradiated F1 hybrid mice that had been injected with a mixture of the parental lymph node and bone marrow cells. In contrast to spleen colonies, which become grossly detectable by the 6th day when the cells are injected into irradiated mice, spleen colonies could not be detected in such nonirradiated F1 hybrid mice before the 16th day. Thus there seems to be a difference in the kinetic features of CFU-S injected into nonirradiated mice from those of CFU-S injected into irradiated mice. In order to clarify the fate of injected CFU-S in nonirradiated mice, a time-course study was carried out and is reported here.

MATERIALS AND METHODS

Mice. BT6F1 (C57BL x CBA-T6T6) hybrid and C57BL mice were used at 2-4 mo of age. C57BL, CBA-T6T6, and BT6F1 mice were raised in our laboratory.

Cell suspensions and irradiation. 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 IU sodium heparin was injected intraperitoneally (i.p.) 20 min before the cell injection. A Toshiba x-ray machine was used to irradiate the animals. The conditions of irradiation were previously described. BT6F1 mice received 850 rads and C57BL mice 790 rads. The cells were injected within 3 hr after irradiation.

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Spleen colony formation in nonirradiated mice. Nonirradiated BT6F1 mice were injected intravenously with spleen cells of immunized C57BL mice that had received two doses of spleen and lymph node cells of CBA-T6T6 mice (20 x 10^6) i.p. at 7-day intervals. The donor spleen cells were obtained from the C57BL mice on the seventh day after the last immunization. Although the mixture of lymph node and bone marrow cells was preferred to spleen cells when the number of injected CFU-S was changed as described in our previous report,6 we used spleen containing both immune cells and CFU-S in the present study because many donor mice are necessary to obtain a sufficient number of lymph node cells.

To confirm the hematopoietic chimerism, chromosome analysis of the cells from the spleen and the femur was carried out using methods described previously.6 In each spleen and femur more than 50 metaphases were scored for the presence or absence of a T6 marker chromosome after confirming that total number of chromosomes was 40.

Histologic examination. The spleen was weighed and fixed with Bouin's solution. Histologic sections were made with conventional technique and stained with hematoxylin and eosin. In order to count microscopic colonies, spleens were removed on the 18th day after injection of parental spleen cells and fixed in Bouin's solution in one experiment. After counting macroscopic colonies with a dissection microscope (X7), the spleen was cut at 50-μm intervals and sections were stained with H&E. Identification and quantification 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 and femur. Spleens and femurs were removed from four to eight nonirradiated BT6F1 mice on different days after injection of the parental spleen cells; the cells from these spleens were suspended in 10 ml Eagle's medium, the cells from femurs in 5 ml medium. A known proportion was injected into 12-15 irradiated C57BL mice. The mice were killed on the eighth day after cell injection. The spleens were removed and fixed in Bouin's solution. Colonies were counted with a dissection microscope (X7). In order to determine the origin of the CFU-S, irradiated C57BL mice that had been immunized with more than three injections of CBA-T6T6 cells were used as recipients. Only the CFU-S of C57BL mouse origin could form colonies in the spleen of such immunized C57BL mice.6

Treatment with 3H-thymidine (3H-TdR). A 3H-TdR "suicide" assay was performed according to the method described by Becker et al.9 Spleen cell suspensions were incubated for 25 min at 37°C with 250 μCi/ml 3H-TdR; the tubes were agitated by hand every 3 min. The control suspensions were incubated at the same time without 3H-TdR. After incubation the cells were washed with ice-cold Eagle's medium.

Granulocyte counts. Blood samples were obtained from a lateral tail vein. Leukocyte counts and differentials were carried out with conventional techniques.

59Fe uptake. The mice were killed 72 hr after the subcutaneous injection of citrated 59Fe (1 μCi), and the 59Fe uptake into erythrocytes was assayed in an auto-well gamma counter. The uptake of 59Fe was expressed as a percentage of the total radioactivity injected. The total uptake was corrected by assuming that the whole blood volume was 6.5% of body weight.10

RESULTS

Spleen colony formation. In nonirradiated BT6F1 mice, which were injected with 10^8 spleen cells from C57BL mice immunized with CBA-T6T6 cells, spleen weights rose to about four times normal on the 10th day (Fig. 1). Thereafter spleen weights decreased to about 1.5 times normal. They began to increase again when hematopoietic colonies appeared on the spleen surfaces (16-22 days after cell injection) (Fig. 1). Histologic examination of the spleens showed the following changes: (1) hypertrophy of the white pulp with active cell divisions during the first 8 days, when spleen weights were increasing; (2) cell division decreased in both white and red pulp from the 10th to the 12th days; (3) hematopoietic foci were observed in the red pulp with active divisions of cells on the 14th day and thereafter. Since typical macroscopic colonies were seen on the
18th day, histologic quantification of colonies was carried out using ten spleens removed on that day: 172 colonies (17.2 ± 1.9, mean ± SE per spleen) were counted macroscopically and 584 colonies (58.4 ± 5.2 per spleen) microscopically. Erythroid colonies (45%) were more numerous than granuloid colonies (22%) and megakaryocytic colonies (25%). Although mixed colonies (8%) were observed, no colonies consisting of only undifferentiated cells were detected.

As shown in Fig. 2, chromosome analysis showed that dividing cells of C57BL mouse origin appeared earlier in the spleen than in the bone marrow and that most of dividing cells were of C57BL mouse origin both in the spleen and in the bone marrow from the 16th day on.

Changes in number of CFU-S. The total number of CFU-S and the number of CFU-S of C57BL mouse origin were determined in the spleen and the femur of the BT6F1 hybrid mice on various days after injection of parental cells. The total number of CFU-S in the spleen started to decrease on the fifth day, dropping to less than 1% of the normal value by the ninth day. Numbers of CFU-S of C57BL mouse origin decreased during the first 9 days as well. The total number of CFU-S in the spleen increased thereafter, with a doubling time of approximately 36 hr. As shown in Fig. 3, CFU-S that appeared in the spleen after the ninth day were those derived from C57BL mice.

The total number of CFU-S in the femur started to decrease on the 8th day and became less than 1% of original levels on the 16th day. The number of
Fig. 3. Number of CFU-S in spleen of BT6F1 mice on various days after injection of spleen cells from C57BL mice. Total number of CFU-S was determined using normal irradiated C57BL mice as recipients. Number of CFU-S of C57BL origin was determined using immunized C57BL mice.

Fig. 4. Number of CFU-S in femur of BT6F1 mice on various days after injection of spleen cells from C57BL mice; total number of CFU-S and number of CFU-S of C57BL origin.
Table 1. Effect of Incubation With $^3$H-Thymidine on Colony-Forming Ability of Cells From the Spleen of BT6F$_1$ Mice

<table>
<thead>
<tr>
<th>Treatment of Primary Hosts</th>
<th>Injection</th>
<th>Mice Used for Colony Assay</th>
<th>No. of Exp.</th>
<th>Suicide Rate (%) (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irradiation</td>
<td>Injected Cells</td>
<td>Days After Injection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No†</td>
<td>None†</td>
<td>—</td>
<td>BT6F$_1$</td>
<td>3</td>
</tr>
<tr>
<td>Yes</td>
<td>$10^8$ BMC of BT6F$_1$</td>
<td>5</td>
<td>BT6F$_1$</td>
<td>4</td>
</tr>
<tr>
<td>No</td>
<td>$10^8$ SC of C57BL</td>
<td>5</td>
<td>Imm. C57BL§</td>
<td>3</td>
</tr>
<tr>
<td>No</td>
<td>$10^8$ SC of C57BL</td>
<td>18</td>
<td>Imm. C57BL§</td>
<td>3</td>
</tr>
</tbody>
</table>

*BMC, bone marrow cells; SC, spleen cells.
†Normal control BT6F$_1$ mice.
§Normal control BT6F$_1$ mice.

The $^3$H-TdR suicide rate of CFU-S of C57BL mouse origin recovered from the spleen of nonirradiated BT6F$_1$ mice was determined on the 5th and 18th days after injection of parental spleen cells. The C57BL mice, which had been immunized against CBA-T6T6 antigens, were used for the assay of spleen colonies. The suicide rate of CFU-S from the spleen of normal BT6F$_1$ mice and that of CFU-S from the spleens of BT6F$_1$ mice that had received irradiation and injection of $10^8$ syngeneic bone marrow cells 5 days before were also determined. The suicide rate of parental CFU-S recovered from the spleen of the nonirradiated chimeras was about 40% on both the 5th and 18th days after cell injection (Table 1).

Changes in peripheral blood. The uptake of $^{59}$Fe into erythrocytes of BT6F$_1$ mice was evaluated on various days after injection of parental spleen cells. The $^{59}$Fe uptake started to decrease from the 7th day, dropping to about 2% of the normal on the 11th day. The $^{59}$Fe uptake began to increase thereafter and rose to the normal level by the 24th day (Fig. 5).

The number of peripheral blood granulocytes increased significantly on the 8th day after the cell injection ($p < 0.001$) and then began to decrease. On the
Fig. 6. Number of peripheral blood granulocytes in BT6F1 mice on various days after injection of parental spleen cells.

17th day the number of granulocytes was about 2% of normal, and the level increased again thereafter (Fig. 6).

DISCUSSION

Hematopoietic spleen colonies developed 16-22 days after injection of parental spleen cells into nonirradiated BT6F1 hybrid mice. This result is consistent with our previous experiment in which a mixture of lymph node and bone marrow cells was used rather than the spleen cells employed in the present experiment.6

The time-course study of the change in the number of CFU-S showed that both CFU-S of F1 hybrid host and of parental mouse origin were decreasing in the spleen of the BT6F1 mice during the first 9 days after the injection of the parental spleen cells. The decrease of the former was probably a direct result of graft-versus-host (GVH) reaction induced by the parental immune cells,6 while the decrease of the latter may have been due to hybrid resistance.6 After the decrease, the CFU-S of F1 hybrid origin did not seem to recover because (1) metaphases of F1 hybrid type were rarely found both in the spleen and in the bone marrow from the 16th day on and (2) CFU-S values assayed in normal C57BL mice, in which CFU-S of both F1 hybrid and C57BL mouse origin formed colonies, were comparable with the CFU-S values assayed in the immunized C57BL mice, in which only the CFU-S of C57BL mouse origin could form colonies, from the 9th day in the spleen and from the 15th day in the femur. On the other hand, the decrease of the parental CFU-S stopped when the hybrid resistance was abolished by the GVH reaction by the 9th day after cell injection.6,11,12 Another explanation for the decrease in parental CFU-S would be that the parental CFU-S were damaged by the cells of F1 hybrid hosts nonspecifically that were activated by the GVH reaction as described by Singh et al.,13 since the F1 hybrid hosts were not irradiated in the present experiment.

The 3H-TdR suicide technique showed that the CFU-S of parental origin were in cycle on both 5th and 18th days after cell injection. Cycling of the
CFU-S on the 5th day seems compatible with the recent hypothesis of Brecher et al. that triggering of CFU-S into cycle after transplantation is necessary to establish them in normal recipients.\(^\text{14}\) Cycling of parental CFU-S seems necessary to repopulate hematopoietic tissues of nonirradiated \(F_1\) hybrid hosts.

The number of parental CFU-S was decreasing on the 5th day in spite of their active cycling. Chervenick and Boggs reported that CFU-S divide solely to replicate themselves and do not differentiate whenever the total number of CFU-S is decreased to less than 10% of normal.\(^\text{3}\) Hence our results may be interpreted as parental CFU-S self-replicating without differentiating for the first 9 days. Although the number of the parental CFU-S started to increase after the 9th day in the spleen of the \(F_1\) hybrid hosts, the doubling time of the CFU-S (about 36 hr) was longer than the time reported in the spleen of lethally irradiated mice (16 hr,\(^\text{3}\) 24 hr\(^\text{4}\)). The retarded start of differentiation and the longer doubling time may well explain the fact that the spleen colonies of parental origin could not be detected before the 16th day in nonirradiated mice.

Uptake of \(^{59}\)Fe into erythrocytes was suppressed as early as eradication of CFU-S from the spleen, probably because of the suppressed differentiation of CFU-S into erythroid precursors in the bone marrow, since there were considerable CFU-S in the bone marrow at that time. The \(^{59}\)Fe uptake increased when the erythroid colonies appeared in the spleen. Histologic studies showed that active division of the cells occurred in erythroid colonies but not in the white pulp or in the red pulp without colonies. The finding thus supports our previous contention that active proliferation of immune cells seems necessary to suppress erythropoiesis.\(^\text{15}\)

A significant granulopoiesis, observed on the eighth day after cell injection, may be the result of enhanced production of colony-stimulating factor (CSF) in \(F_1\) hybrid hosts with GVH reaction as previously described by Hara et al.\(^\text{16}\) The granulocytopenia that followed the granulocytosis was probably due to the eradication of CFU-S from the bone marrow, the principal site of granulocyte production.\(^\text{17}\)

In conclusion, it is suggested that CFU-S are stimulated into proliferation to repair the decrease of CFU-S caused by immune reactions between host and donor cells; that donor CFU-S replace host CFU-S during the first 9 days after injection of parental spleen cells; and that subsequently the donor CFU-S can initiate growth like CFU-S injected into lethally irradiated mice. The use of the GVH reaction provides a new technique for the kinetic study of stem cell proliferation.

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