T- and B-Lymphocyte Differentiation Potentials of Spleen Colony-Forming Cells

By Françoise Lepault, Sophie Ezine, and Marie-Claude Gagnerault

Cells that generate splenic colonies within 8 days (day-8 colony-forming units spleen [CFU-s]) are generally thought to differentiate only into erythroid/myeloid cells. The T and B lymphocyte differentiation potentials of day-8 CFU-s were evaluated and compared with those of day-12 and 5-fluorouracil (5-FU) CFU-s. This was achieved by analyzing, after intravenous and intrathymic injection, the lymphocyte progeny of cells contained within individual splenic colonies collected at day 8 and day 12 post-bone marrow cell transfer into irradiated congenic recipients. A large majority of day-8 spleen colonies generated T cells when transferred intrathymically. After intravenous (IV) injection of day-8 colonies, donor-type thymocytes emerged in 33% of the animals reconstituted with only 1 day-8 colony, but in 83% of those inoculated with a pool of 5 colonies. All post-5-FU and 75% of day-12 colonies gave rise to thymocytes after IV injection. B cells were generated by a high proportion of day-8 colonies, and by all day-12 and post 5-FU colonies. These results demonstrate that progenitors of T and B lymphocytes are generated within spleen colonies produced by at least some day-8 CFU-s and virtually all day-12 CFU-s. Whether these progenitors are CFU-s themselves or committed precursors remains an open question.

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BLOOD CELLS of all lineages arise from a common ancestor, a multipotent stem cell. Analysis of criteria such as proliferative capacity, propensity for replication, differentiation potentials, and ability to allow long-term survival of irradiated animals led to the notion of hierarchy within the stem cell compartment.  

The cells that develop as colonies in the spleen of lethally irradiated mice (colony-forming units spleen [CFU-s]) are heterogeneous. Two major subsets have been defined on the basis of the time required for the appearance of macroscopic nodules at the surface of the spleen.  

Late spleen colonies, counted at day 12, originate from CFU-s that are more primitive (with a high self-renewal potential and able to differentiate along all blood cell lineages) than those giving rise to transient colonies, visible at day 8 but not at day 12.  

The capacity of day-12 CFU-s to differentiate along the lymphoid lineages has been established using two different approaches. Wu and Liu,  

using sex chromosome typing after transfer of single spleen colonies into secondary recipients, demonstrated the presence of donor-type T and B lymphocytes in peripheral organs. More recently, cell suspensions highly enriched for day-12 CFU-s were studied. On the other hand, the analysis of the differentiation potentials of day-8 CFU-s has been hampered by the apparent difficulty of purifying these cells without significant contamination by cells giving rise to colonies that persist or develop after day 8. However, based on histologic and myeloid progenitor cell content analyses of splenic colonies,  

cells, whereas the two other congenic strains were used as donors.  

Animals. Congenic female and male mice of the C57BL/Ka Thy-1.2, C57BL/Ka Thy-1.1 strains and F1 (Thy-1.1 × Thy-1.2) mice were provided by Dr W. van Ewijk (Erasmus University, Rotterdam, The Netherlands). C57BL/Ka Thy-1.1 (Ly 5.1) were used as recipients of BM or splenic colony cells, whereas the two other congenic strains were used as donors. All mice were 8 to 10 weeks old.

Preparation of cell suspensions. Single cell suspensions were prepared in RPMI 1640 culture medium; 2% fetal calf serum and 5 mmol/L sodium azide were added for immunofluorescence staining. Before cell labeling, red blood cells were removed from BM and spleen cell suspensions by hypotonic lysis.

5-FU treatment. Donor mice received 150 mg/kg 5-FU intravenously (IV). Three days later, they were killed and pooled BM cell suspensions were prepared.

Antibodies. Fluorescein isothiocyanate (FITC)-anti-Thy-1.2 (clone 30-H 12),  

and bimot anti-Thy-1.1 (clone 19XE5).  

FITC-anti-B20 (clone RA3-6B2).  

and biotin-anti-Ly 5.2 (clone A-20-1.7)  

monoclonal antibodies (MoAbs) and phycoerythrin (PE)-streptavidin (Caltag, South San Francisco, CA) were used for cell staining. Surface markers were analyzed with a FACScan (Becton Dickinson, Grenoble, France).
Experimental protocol. To test whether CFU-s can differentiate along the lymphoid lineages, individual splenic colonies collected at days 8 and 12 were used to repopulate secondary irradiated animals upon IV or intrathymic (IT) injection. Various times after the reconstitution, the presence of donor-type lymphocytes in the recipients’ organs was determined.

Preparation of spleen colonies. Spleen colonies were prepared as described by Till and McCulloch. 16 Briefly, BM cells from normal (4 × 10^4) or 5-FU-treated (5 × 10^4) donors were injected IV into irradiated (8.5 Gy, 50 Co total body irradiation) congenic recipients. Eight to 12 days later, mice were killed, their spleens excised, and nodules were individually enucleated under binocular lenses. To minimize possible contamination by surrounding splenic tissue, colonies grown at the edge of the spleen were preferentially harvested. Each nodule was disrupted with a Pasteur pipet, and cells were washed once and resuspended in 0.3 mL culture medium for IV injections and in 20 μL for IT transfers. Day-8 and -12 colonies were generated from colonies grown at the edge of the spleen were preferentially harvested. Eight to 12 days later, mice were killed, their spleens excised, and nodules were individually enucleated under binocular lenses. To minimize possible contamination by surrounding splenic tissue, colonies grown at the edge of the spleen were preferentially harvested. Each nodule was disrupted with a Pasteur pipet, and cells were washed once and resuspended in 0.3 mL culture medium for IV injections and in 20 μL for IT transfers. Day-8 and -12 colonies were generated from colonies grown at the edge of the spleen were preferentially harvested. Each nodule was disrupted with a Pasteur pipet, and cells were washed once and resuspended in 0.3 mL culture medium for IV injections and in 20 μL for IT transfers. Day-8 and -12 colonies were generated by normal BM cells; post-5-FU colonies were harvested only on day 12. More than 180 spleen colonies were tested in this study.

Reconstitution of secondary recipients. Cells from a single nodule were injected, either IV or IT, into one secondary lethally irradiated congenic recipient. In some experiments, cells from 2 to 5 nodules were pooled before IV transfer. Recipients were rescued by an IV injection of 1 to 2 × 10^6 isogenic BM cells (C57BL/Ka Thy-1.1), except those that were reconstituted IV with a spleen colony generated by post-5-FU BM cells. This injection was performed 24 hours after colony cell injection to avoid competition between the two cell types for early homing. At appropriate intervals, recipients were killed and cell suspensions from thymus, spleen, mesenteric lymph node (MLN), and BM were prepared for immunofluorescence analysis. When donors and recipients were congenic at the Thy-1 locus, donor-type T cells were discriminated from the recipient cells after double staining with FITC-anti-Thy-1.2 and biotin-anti-Thy-1.1 MoAbs. Mouse congenic at both the Thy-1 and Ly 5 loci, allowed us to distinguish donor-type T (Thy-1.2+, Ly 5.2+) and B (B220+, Ly 5.2+) cells. A recipient’s organ that contained at least 0.1% brightly stained donor-type cells was considered positive. Negative animals and syngeneic controls contained ≤0.02% cells expressing such high fluorescence intensity.

IT transfer assay. All or half of the cells from a single splenic nodule were injected into each thymic lobe of a recipient, under ether anesthesia. 17 When 5-FU colonies were very large, only one-fourth of their content was transplanted into each lobe. Recipients were sublethally irradiated (6.5 Gy total body irradiation) 3 to 4 hours before surgery. They were killed 3 to 4 weeks later, and a cell suspension was prepared from each thymus. In some experiments, cells from a whole nodule were injected into one lobe. In the second lobe, used as negative control, no cells were found to be as brightly stained as donor-type cells were.

Clonality of spleen colonies. To determine whether reconstitution of secondary recipients is derived from the progeny of the colony-forming cell, or from immature hematopoietic stem cells or lymphocyte precursors that do not form colonies but may contaminate the nodules, we performed two control experiments. If such cells were to seed the spleen and remain in their initial differentiation stage, it is likely that they would be localized randomly, ie, inside as well as outside the colonies. Therefore, intercolony spaces were tested for donor-type reconstitution ability. Cell suspensions of pieces of spleen tissue, with the average size of a large colony, harvested between day-12 and 5-FU colonies were injected to irradiated recipients. Their thymi were analyzed 3 to 4 weeks later.

Second, a 1 to 1 mixture of BM cells from congenic Thy-1.1 and Thy-1.2 mice was injected into irradiated F1 (Thy-1.1 × Thy-1.2) primary recipients. Twelve days later, the spleen colonies were harvested and injected into irradiated F1 secondary recipients. The presence of donor-type thymocytes was determined 3 to 4 weeks later using double immunofluorescence staining for Thy-1.1 and Thy-1.2 alleles. Cells generated by the injected colony cells express either one of the Thy-1 markers, whereas recipient thymocytes express both alleles (Fig 1). If spleen colonies were contaminated by cells with pre-T activity, the latter would be of either donor type. Thus, statistically one-half of the contaminates colonies would contain cells having a Thy-1 marker identical to that of the colony founder, and contamination would not be detected in secondary recipients. However, the rest of the colonies would contain cells with a different Thy-1 marker whose progeny would be detected in secondary recipients.

RESULTS

Generation of spleen colonies. The frequency of CFU-s was on average 20/10^4 in normal BM and 2/10^5 in 5-FU-treated BM. The average size of the colonies increased from day 8 to day 12, but small colonies were visible on day 12 and colonies of various sizes were enucleated from the spleen.

Contamination of colonies by immature stem cells is unlikely. It was important to document that donor-type cells in secondary recipients were derived from the same cell that gave rise to the spleen colony. First, no cells with repopulation properties were found between day-12 colonies generated by normal BM (10 intercolony spaces from 5 spleens were tested), as previously reported, 6 or 5-FU-treated BM (7 intercolony spaces tested). Second, a mixture of BM cells from mice congenic at the Thy-1 locus generated colonies into F1 recipients; upon IT injection, 6 of 7 of these day-12 colonies gave rise to only one type of donor cells. The proportion of donor-type cells ranged from 0.5% to 59% of total thymocytes. No donor-derived thymocytes were generated by 1 of the 7 colonies. A second experiment showed that in three F1 mice reconstituted IV with 1 day-12 colony, Thy-1.1 thymocytes represented 0.4%, 0.7%, and 6% of total thymocytes, and no Thy-1.2" were detectable. In both experiments, the rest of the thymocytes expressed both alleles (F1 phenotype) besides...
rare cells that were Thy-1\(^+\) (Fig 1). Thus, an individual colony appears to be not contaminated by cells with pre-T activity that were not generated by that clone.

**Early as well as late hematopoietic spleen colonies contain cells able to differentiate along the T-cell lineage.** When normal BM cell-derived colonies (Thy-1.2) were injected IV into sublethally irradiated recipients (Thy-1.1), donor-type thymocytes were detected from the third week posttransfer. A discrete thymocyte population appeared in about 30% of the recipients reconstituted with 1 to 3 day-8 colonies 3 to 4 weeks earlier. However, injection of 5 day-8 colonies allowed the generation of a higher proportion of thymocytes in 83% of the hosts. This thymic population seems to be transient because it was no longer observed at weeks 5 to 8 (Table 1 and Fig 2).

Injection of a single day-12 colony was sufficient to allow the regeneration of the thymus in 75% of the recipients at weeks 3 to 4. Reconstitution of mice with 2 colonies led to the generation of a higher mean percentage of thymocytes in 100% of the animals.

Post-5-FU colonies contain cells that gave rise to donor cells in 71% of the recipients as early as 10 days postinjection. Later, all hosts were positive for donor-type Thy-1.2\(^+\) thymocytes (Table 1).

Interestingly, 76% of the animals transferred IT with a single day-8 colony developed donor-type thymocytes (Fig 2). Upon IT transfer, all day-12 and 5-FU colonies tested gave rise to Thy-1.2\(^+\) thymocytes (Table 1).

Donor-type Thy-1.2\(^+\) thymocytes expressed CD4 and CD8 antigens whether they were the progeny of cells transferred IV or IT (data not shown). In addition, Thy-1.2\(^+\) cells were able to migrate out of the thymus because donor-type lymphocytes were found in peripheral lymphoid organs. After the transfer of 5-FU colonies, donor-type lymph node T cells were detectable from the second week in 100% of host mice (data from week 2 not shown), and their frequencies were higher than in mice reconstituted with normal BM-derived colonies (Table 2).

**The three types of CFU-s tested have the capacity to differentiate along the B-cell lineage.** Production of donor-
type Ly 5.2+ cells in BM, spleen, and MLN was studied together with the generation of B cells (Ly 5.2+ B220+) (Table 3).

When day-8 and day-12 colonies generated by Ly 5.2+ BM cells were transplanted into Ly 5.1 congenic recipients, donor-type B cells were detectable in the three organs 3 to 4 weeks later.

Interestingly, after 1 day-8 colony injection, donor-type cells and B cells were detected in the BM of only 1 of 8 animals (12%), whereas virtually all the lymphoid organs contained such cells. When more than 1 day-8 colony was injected, the proportion of donor B cells increased in peripheral organs.

Post-5-FU colonies allowed the production of B cells in the three organs tested in all the animals. It is interesting to note that not only the mean fraction of donor-derived B cells was higher than that observed in animals reconstituted with the two other types of colonies, but in some recipients the frequency of these cells was comparable to that found in nonirradiated mice.

DISCUSSION

The major conclusion arising from the results presented herein is that early as well as late spleen colonies contain cells capable of differentiating along both B- and T-cell lineages. Given the clonal origin of these colonies, these observations indicate that the majority of CFU-s are multipotent.

When unseparated BM cells are injected into irradiated animals, around 50% of the colonies visible at day 8 have disappeared by day 12, and have been replaced by an approximately equal number of colonies. Thus, about only one-half of day-8 colonies arise from day-8 CFU-s, and approximately one-half of day-12 colonies are persistent, whereas the second half comprises delayed colonies generated by true day-12 CFU-s. Because 33% of the colonies harvested at day 8 (individually injected IV) gave rise to thymocytes, it cannot be excluded that these colonies would have persisted until day 12; thus, these thymocytes may not arise from true day-8 CFU-s. However, 76% of day-8 colonies injected directly into the thymus exhibited a pre-T-cell activity. This frequency is too high to represent differentiation toward T lymphocytes of only persistent colony formers. This indicates that approximately 25% of day-8 colonies are generated by day-8 CFU-s that can give rise to T cells; thus, approximately 1 of 2 day-8 CFU-s has a pre-T-cell activity. IT transfer can detect subsets of nonmigrating as well as migratory thymocyte precursors. Although it is clear that stem cells can differentiate into T cells when transplanted into the thymus, it is not yet established whether the pre-T cell that homes to the thymus under physiologic conditions is a stem cell or a committed precursor (prothymocyte). Therefore, the loss of homing property of day-8 CFU-s or their progeny could explain the poor thymic repopulation observed after IV transfer of individual day-8 colonies. Alternatively, day-8 colonies may not contain a sufficient number of pre-T cells so that, upon IV injection, given their low seeding efficiency, at least one of them could colonize the thymus. Indeed, reconstitution with a pool of 5 colonies led to the production of donor-type thymocytes in more than 80% of the recipients and the average frequency of Thy-1.2 cells per organ increased about 30-fold as compared with that of recipients of a single colony. Thus, donor-type cells are technically detectable if a minimum number of T-precursor cells is infused. This suggests that the frequency of 33% colonies containing pre-T cells was underestimated. Populations enriched for day-8 CFU-s, but still containing a large proportion of cells generating colonies visible at day 12, failed to form thymocytes upon IT3 or IV3.7 injection. Phenotype differences, used as discriminatory parameters in cell sorting, may divide day-8 colony formers into subsets having distinct differentiation potentials. In addition, cell sorting allows collection of only a fraction of the CFU-s contained in total BM.

Donor-type T cells were detected in the lymph nodes of most but not all day-8 colony recipients whose thymus contained donor-derived thymocytes. The frequency of the latter was so low that thymus migrants were most likely too diluted to be detected in the MLN of some recipients. Seventy-five percent of day-8 colonies generated B cells detectable in the lymphoid organs; thus, the majority of day-8 CFU-s have a pre-B-cell activity. These results suggest that seeding of BM by cells having a pre-B-cell activity is more efficient than that of the thymus, and/or that day-8 CFU-s generate more B-cell precursors than T-cell precursors during colony growth. Only 1 of 8 animals reconstituted with a single day-8 colony

| Table 2. Donor-Type T Cells in the MLN of Secondary Recipients 3 to 5 Weeks After Reconstitution With Splenic Colonies |
|-------------------------------------------------|------------------|------------------|------------------|
| **Spleenic Colonies**                          | **Day-8**        | **Day-12**       | **Post-5-FU**    |
| **Weeks After Transfer**                       | **Route of Injection** | **No. of Injected Colonies** | **Positive Mice/Total** | **Mean* (range)** | **Positive Mice/Total** | **Mean* (range)** | **Positive Mice/Total** | **Mean* (range)** |
| 3-5                                            | IV               | 1                | 2/7              | 0.1              | 10/13             | 0.8              | (0.1-6)              | 23/23             | 26.4              | (6-68)             |
|                                                | 2                |                  |                  |                  | 10/10             | 8.6              | (1-21)              |                  |                   |                    |
| 4-5                                            | IT               | 0.5-1            | 7/10             | 8.9              | 1/7               | 6                | (0.1-45)             |                   | 8/9               | 2.5                | (1-6)              |
|                                                |                  |                  |                  |                  |                   |                   |                      |                   |                   |                    |

* Mean percentage of Thy 1.2+ cells per MLN in positive mice.
<table>
<thead>
<tr>
<th>Host’s Organ</th>
<th>No. of Colonies Injected</th>
<th>Ly 5.2+ ( ^{\dagger} )</th>
<th>% Range of Positive Cells</th>
<th>Ly 5.2+ ( B_{220}^{+} ) ( ^{\ddagger} )</th>
<th>% Range of Positive Cells</th>
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<tr>
<td>BM</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Day-8</td>
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<td>1/8</td>
<td>0.1</td>
<td>1/8</td>
<td>0.1</td>
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<tr>
<td>Day-12</td>
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<td>0.2-3.1</td>
<td>10/12</td>
<td>0.1-0.2</td>
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<tr>
<td>Post-5-FU</td>
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<td>13/13</td>
<td>80-97</td>
<td>13/13</td>
<td>3-16</td>
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<tr>
<td>Spleen</td>
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</tr>
<tr>
<td>Day-8</td>
<td>1</td>
<td>8/8</td>
<td>0.1-02</td>
<td>7/8</td>
<td>0.1-0.2</td>
</tr>
<tr>
<td>Day-12</td>
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<tr>
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<td>6/8</td>
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<td>17/18</td>
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<td>11/11</td>
<td>20-50</td>
<td>10/10</td>
<td>3.5-30</td>
</tr>
</tbody>
</table>

\( ^{\dagger} \) Recipients were killed 3 to 4 weeks after their reconstitution.

\( ^{\ddagger} \) Total donor-type cells.

\( ^{\ddagger} \) Donor-type B cells.

had donor-type \( B_{220}^{+} \) cells in the BM. Because B-cell production does not take place in the spleen\(^{21}\) it is likely that peripheral donor-type B cells observed in animals with no medullary \( B_{220}^{+} \) cells of donor origin have migrated from the BM. This suggests that day-8 CFU-s rapidly lose their lymphocyte differentiation potential when they are induced to proliferate and differentiate, and generate B precursors with low or no self-renewal capacity. In support of this hypothesis is the transitory presence of donor-type T cells in the thymus of recipients injected IV with day-8 colonies.

Day-12 CFU-s exhibited a pre-T activity in all cases when their progeny was transplanted IT, and in 75% of cases after IV injection. The donor-type cells represented a higher proportion of thymus and MLN cells than in recipients of day-8 colonies. B cells produced by day-12 colonies were detected in the three organs of virtually all mice tested.

The antitumor agent 5-FU spares primitive stem cells with marrow repopulating ability and day-12 CFU-s, but drastically reduces the number of day-8 CFU-s.\(^{10,22}\) All colonies generated by post-5-FU CFU-s contained both pre-T and pre-B activities. It is noteworthy that donor-type thymocytes could be detected as early as 10 days after IV transfer of a single post-5-FU colony in 70% of the recipients. This frequency was reached only 3 to 4 weeks after injection of late colonies generated by normal BM. In the second week, all recipients of post-5-FU colonies were positive for donor-derived thymocytes. The numbers of the latter were generally higher in recipients of post-5-FU colonies than after normal day-12 colony injection, but their range was wide for both colony types. Post-5-FU colonies formed B cells in 100% of recipients tested, with frequencies higher than those observed with the other colony types.

Because the time course of thymus reconstitution depends on the number of cells with pre-T activity that are injected,\(^{23}\) our results suggest that post-5-FU colonies contain a higher level of such precursors than the colonies generated by 5-FU-sensitive cells. Likewise, spleen colonies containing the highest concentrations in CFU-s are those generated by 5-FU BM cells,\(^{24}\) indicating their extensive self-renewal capacity.\(^{10}\) The reconstitution of the thymus starts later after injection of post-5-FU BM cells than after injection of normal BM cells, suggesting that stem cells surviving to 5-FU need to further differentiate to express T-lymphocyte differentiation potential.\(^{7}\) Taken together, this observation and our results suggest that such differentiation steps occur during colony growth and result in the emergence of cells able to rapidly respond to the proliferation and differentiation signals provided by the thymic microenvironment. Hence, the thymus-homing spleen colony cell is probably not the pre-CFU-s spared by 5-FU treatment, but whether it is a multipotent stem cell or a committed prothymocyte remains to be established. Recent work\(^{25-28}\) reopened the issue of whether day-8 and day-12 CFU-s represent different stem cell types, as was originally proposed.\(^{2}\) Our results give evidence that, with regard to their differentiation potentials, some day-8 CFU-s are not distinct from day-12 CFU-s, but are multipotent.

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REFERENCES


4. Ploemacher RE, Brons NHC: Cells with marrow and spleen repopulating activity and forming spleen colonies on day 16, 12 and 8 are sequentially ordered on the basis of increasing rhodamine 123 retention. J Cell Physiol 136:531, 1988

5. Ploemacher RE, Brons MHC: Isolation of hemopoietic stem cell subsets from murine bone marrow: I. Radioprotective ability of purified cell suspensions differing in the proportion of day-7 and day 12 CFU-S. Exp Hematol 16:21, 1988


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