Lympoid Differentiation of the Hematopoietic Stem Cell That Reconstitutes Total Erythropoiesis of a Genetically Anemic W/W' Mouse

By Toru Nakano, Noriko Waki, Hidekazu Asai, and Yukihiko Kitamura

We investigated whether the stem cell that reconstitutes total erythropoiesis of a WBB6F1-W/W' mouse differentiates into lymphoid lineage. The electrophoretic pattern of hemoglobin was used as a marker of the reconstitution: 3-phosphoglycerate kinase (PGK), an X chromosome-linked enzyme was used as a tool for estimating clonality. We injected 10^5 bone marrow cells of 5-FU treated C57BL/6-Pgk-1b/Pgk-1b female mice, in each which stem cell had either A-type PGK or B-type PGK due to random inactivation of one of two X chromosomes, into genetically anemic (WB x C57BL/6)F1-W/W' (hereafter WBB6F1-W/W' ) mice that contained only B-type PGK. The recipient WBB6F1-W/W' mice, in which erythropoiesis was reconstituted with donor cells for a long term, were killed and the PGK patterns of bone marrows, thymus, lymph nodes, and Peyer's patches were examined. A considerable amount of A-type PGK was detected in the lymphoid organs of the WBB6F1-W/W' mice in which erythrocytes showed only A-type PGK when killed. In contrast, A-type PGK was scarcely detectable in the lymphoid organs of the WBB6F1-W/W' mice in which erythrocytes showed only B-type PGK when killed. The present results suggest that the hematopoietic stem cells estimated by the erythropoiesis reconstituting assay differentiate into lymphoid lineage and that the long-term erythropoiesis reconstitution assay is useful for detecting the true primitive hematopoietic stem cells.

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The hematopoietic stem cell is characterized by its extensive self-maintaining capacity and differentiation potential.1,3 The hematopoietic stem cell that forms a macroscopic colony in the spleen of either lethally irradiated or genetically anemic (WB x C57BL/6)F1-W/W' (hereafter WBB6F1-W/W') mice-2 colony-forming units in spleen (CFU-S) is not considered to have extensive self-maintaining capacity; since bone marrow cells containing 5 to 50 CFU-S are necessary to rescue anemia of WBB6F1-W/W' mice.4 Recently we investigated which is more important for the long-term reconstitution of erythropoiesis: the transplantation of multiple CFU-S or that of a single stem cell with extensive self-maintaining capacity.6 We found that the transplantation of a single stem cell with extensive self-maintaining capacity was important for the reconstitution and that the concentration of such a stem cell was 1 per 1.4 x 10^5 5-fluourouracil (5-FU)-treated marrow cells. In the present study, the electrophoretic pattern of 3-phosphoglycerate kinase (PGK), an X chromosome-linked enzyme, was used to estimate the number of stem cells necessary for erythroid reconstitution; C57BL/6-Pgk-1b/Pgk-1b mice were used as donors of bone marrow cells at 2 to 3 months of age. The A-type/B-type ratio of PGK in erythrocytes of C57BL/6-Pgk-1b/Pgk-1a mice is about 50/50. WBB6F1-W/W' mice were used as recipients at 4 months of age. Hemoglobin of WBB6F1-W/W' mice (Hbb^a/Hbb^b) can be distinguished from that of C57BL/6-Pgk-1b/Pgk-1a mice (Hbb^b/Hbb^b) by electrophoresis.16,17

5-FU treatment and bone marrow transplantation. Hematopoietic stem cells surviving the 5-FU treatment are reported to have more extensive proliferative potential than nontreated stem cell populations.16,17 5-FU (150 μg/g body weight) was injected intravenously (IV) to C57BL/6-Pgk-1b/Pgk-1b mice; the mice were killed four days after the 5-FU injection, and bone marrow cells were collected from five mice and suspended in Eagle's medium according to the method previously described.18

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Submitted August 10, 1988; accepted November 29, 1988.

Supported by grants from the Ministry of Education, Science and Culture, the Ministry of Health and Welfare, the Hoansha Foundation, and the Cell Science Research Foundation.

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0006-4971/89/7305-0033$3.00/0
Table 1. Classification of WBB6F1-W/W mice whose hemoglobin type was changed to donor type

<table>
<thead>
<tr>
<th>Group</th>
<th>PGK Pattern</th>
<th>No. of Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Throughout A</td>
<td>1</td>
</tr>
<tr>
<td>A</td>
<td>AB to A</td>
<td>6</td>
</tr>
<tr>
<td>B</td>
<td>Throughout B</td>
<td>2</td>
</tr>
<tr>
<td>B</td>
<td>AB to B</td>
<td>4</td>
</tr>
<tr>
<td>A</td>
<td>Throughout AB</td>
<td>16</td>
</tr>
</tbody>
</table>

NOTE. 10 mice bone marrow cells of 5FU-treated C57BL/6-Pgk-1*/Pgk-1* mice were injected into 200 rad irradiated WBB6F1-W/W mice. In total, 58 WBB6F1-W/W mice survived at least 16 weeks after the bone marrow transplantation; in 29 mice, long-term reconstitution occurred.

Recipient WBB6F1-W/W mice were irradiated (200 rad) with a Shimadzu X-ray machine (180 kV and 20 mA, with 2 mm Al filter, 50 rad/min) to facilitate the bone marrow reconstitution. 5-FU-treated bone marrow cells (10^7) were injected through the lateral tail vein.

Sampling of blood and examination of hemoglobin. Blood samples were collected from retroorbital sinus and packed erythrocytes were obtained as previously described. Electrophoresis of samples were collected from retroorbital sinus and packed erythrocytes were obtained as previously described.6 Electrophoresis of samples were collected from retroorbital sinus and packed erythrocytes were obtained as previously described.

Hemoglobin was done after modification with cystamine according to the method described by Reddy and Falikow.2 Reddy and Falikow,2 the relative activity of PGK alloenzymes was measured.

Electrophoresis was carried out on a gel plate according to the method described by Tanooka and Tanaka.22 According to the report of Reddy and Falikow,2 the relative activity of PGK alloenzymes was measured.

By this method, hemoglobin of WBB6F1-W/W mice (Hbb'/Hbb) mixed with hemoglobin of C57BL/6-Pgk-1*/Pgk-1* mice (Hbb'/Hbb) is detectable if the former contributes 5% of the total. We assumed that WBB6F1-W/W recipient mice were reconstituted by bone marrow cells of C57BL/6-Pgk-1*/Pgk-1* mice only when the hemoglobin pattern of the former could not be distinguished from that of the latter. In other words, >95% of erythrocytes in the reconstituted WBB6F1-W/W mice were derived from the bone marrow of C57BL/6-Pgk-1*/Pgk-1* mice.

PGK electrophoretic analysis of blood samples and tissue samples. Blood samples (packed erythrocytes) were taken, as described above, stored at -80°C. After thawing, cell pellets were put on small pieces of filter paper and inserted into the gel plate. The electrophoresis was carried out on a gel plate according to the method described by Tanooka and Tanaka.22 According to the report of Reddy and Falikow,2 the relative activity of PGK alloenzymes was measured.

In the WBB6F1-W/W mice in which erythropoiesis had been reconstituted for more than 8 weeks and erythrocytes showed only A-type PGK or only B-type PGK, the following tissue samples were taken; bone marrows (both femora, both tibiae, and both humeri), spleen, thymus, lymph nodes (inguinal, axillary, cervical, and mesenteric), and Peyer's patches. Tissue were dissociated into single-cell suspensions in Eagle's medium and filtered through nylon mesh. Contaminating erythrocytes were lysed by osmotic shock with NH4Cl. After washing, the cells were pelleted and frozen at -80°C.

The electrophoresis was carried out as described above, and the percentage of A-type PGK was determined. An average of the percentages of both femora, both tibiae, and both humeri was used as the proportion of A-type PGK in the bone marrow. In other words, the percentages in inguinal, axillary, cervical, and mesenteric lymph nodes was used as the proportion of A-type PGK in the lymph node.

In vitro colony formation and PGK electrophoresis of colonies. Bone marrow cells (2 x 10^6) of the right femur of each reconstituted mouse were plated in 35-mm plastic dishes (Falcon, Beckton-Dickinson, Cocksley, MD) in 1 mL of a-medium (Sigma Chemical, St Louis) containing 30% fetal calf serum (Hyclone Lab, Logan, UT), 0.8% methylcellulose (Sigma, 1% deionized bovine serum albumin (Sigma), 10^-4 mol/L 2-mercaptoethanol (Sigma), 2.0 U/mL of step III sheep plasma erythropoietin (Connaught Lab, Willowdale, Ontario, Canada), and 200 U Interleukin-3 provided by Dr Ihle of the National Cancer Institute. The cultures were incubated for ten days in a CO2 incubator to develop colonies. For the electrophoresis, about 20 erythroid and 20 nonerythroid colonies were lifted up with a 3-µL Eppendorf micropipette under the inverted microscope, and individual colonies were resuspended in 150-µL of a-medium; 50 µL of cell suspensions were used for morphologic identification of constituting cells in individual colonies; and the remaining 100 µL of the suspensions were centrifuged and the cell pellets were frozen in -80°C. After thawing, pellets were put on cellulose acetate membrane and the electrophoresis was carried out on cellulose acetate membrane (Sepaphore III, Gelman Sciences Inc, Ann Arbor, MI) according to the method described by Bücher et al.22

RESULTS

Bone marrow cells (10^7) of 5FU-treated C57BL/6-Pgk-1*/Pgk-1* mice were injected into 200 rad irradiated WBB6F1-W/W mice. In total, 58 WBB6F1-W/W mice survived at least 16 weeks after bone marrow transplantation; in 29 mice, a pure Hbb'/Hbb hemoglobin pattern was maintained at least for 8 weeks. The PGK electrophoretic pattern

Table 2. Proportion of A-type PGK in various hematopoietic and lymphoid organs of the reconstituted WBB6F1-W/W mice in which erythrocytes showed only A-type PGK when killed (Group A)

<table>
<thead>
<tr>
<th>Time Kill After Bone Marrow Transplantation (Wk)</th>
<th>Duration of Reconstitution with Erythrocytes of A-Type PGK Alone (Wk)</th>
<th>Percentage of A-Type PGK in Described Organs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Erythrocytes</td>
<td>Bone Marrow</td>
</tr>
<tr>
<td>A-1*</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>A-2†</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>A-3†</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>A-4†</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>A-5†</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>A-6†</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>A-7†</td>
<td>100</td>
<td>80</td>
</tr>
</tbody>
</table>

Abbreviation: NT = not tested.

* The PGK pattern of this mouse is 'throughout A' in Table 1.
† The PGK pattern of this mouse is 'AB to A' in Table 1.
of erythrocytes was examined in these 29 erythropoiesis-reconstituted WBB6F1-W/Wv mice, and then these mice were divided into three groups with the PGK pattern as a criterion (Table 1). In Group A, erythrocytes of the WBB6F1-W/Wv mice showed only A-type PGK finally. Erythrocytes showed only A-type PGK throughout the reconstitution in one WBB6F1-W/Wv mouse (Table 2) and erythrocytes showed AB-type PGK at the beginning of reconstitution in six WBB6F1-W/Wv mice (Table 2). In Group B, erythrocytes of the WBB6F1-W/Wv mice showed only B-type PGK finally. Erythrocytes showed only B-type PGK throughout the reconstitution in two WBB6F1-W/Wv mice (Table 3) and erythrocytes showed AB-type PGK at the beginning of reconstitution in four WBB6F1-W/Wv mice (Table 3). In Group AB, erythrocytes showed AB-type PGK throughout the reconstitution period.

The WBB6F1-W/Wv mice in Group A and Group B were killed 16 to 40 weeks after the bone marrow transplantation and percentages of A-type PGK of bone marrows, spleen, thymus, lymph nodes, and Peyer’s patches were examined. Figure 1 shows PGK electrophoretic patterns of various organs from a representative mouse in Group A. A considerable amount of A-type PGK was detectable in the organs of the WBB6F1-W/Wv mice in Group A (Table 2). In contrast, A-type PGK was scarcely detectable in the organs of the WBB6F1-W/Wv mice in Group B (Table 3).

Although the percentages of A-type PGK in erythrocytes were 100% in the WBB6F1-W/Wv mice of Group A, the bone marrow contained only small amounts of B-type PGK (Table 2). The organs of these mice could be divided into three groups with the percentage of A-type PGK; bone marrow and thymus showed high value, spleen showed middle value, and lymph node and Peyer’s patch showed low value.

Bone marrow cells obtained from the right femur of the WBB6F1-W/Wv mice in Group A or Group B were cultured in methylcellulose, and PGK types of individual erythroid and nonerythroid colonies were determined. Proportions of erythroid and nonerythroid colonies with A-type PGK were comparable to the percentage of A-type PGK in bone marrow cells of the right femur that were cultured to produce colonies (Table 4). There were no significant differences between the proportion of erythroid colonies with A-type PGK and that of nonerythroid colonies with A-type PGK. Morphologic examination revealed that both erythroid and nonerythroid colonies contained neutrophils, macrophages, megakaryocytes, and mast cells.

### DISCUSSION

In our previous study, we showed that the concentration of the hematopoietic stem cell that reconstituted total erythropoiesis of a WBB6F1-W/Wv mouse was one per $1.4 \times 10^4$ (95% confidence limit was 1.1 to $1.9 \times 10^4$) bone marrow cells of 5-FU-treated C57BL/6-Pgk-1* Pgk-1* mice. In this study, $10^5$ bone marrow cells of 5-FU-treated C57BL/6-Pgk-1* Pgk-1* mice were injected, and the proportion of the erythropoietic reconstitution was 29 of 58 ($=0.5$). This proportion is consistent with our previous data. The probability (P) that $10^5$ 5-FU–treated bone marrow cells contain two or more hematopoietic stem cells that can reconstitute total erythropoiesis of a WBB6F1-W/Wv mouse is calculated as $0.15$ by Poisson distribution ($P = 1 - e^{-10^5} - 1.0/1.4 \times e^{-10^5}$, since $1.4 \times 10^4$ 5-FU–treated bone marrow...
cells contain one hematopoietic stem cell that can reconstitute total erythropoiesis and $1.0 \times 10^3$ 5-FU-treated marrow cells were injected in the present study). In other words, when $10^3$ 5-FU-treated bone marrow cells are injected, about 70% of the reconstituted WBB6F1-W/W' mice are considered to receive only one such hematopoietic stem cell.

The electrophoretic pattern of PGK was throughout A or B in three reconstituted WBB6F1-W/W' mice, and was "AB to A" or "AB to B" in ten reconstituted WBB6F1-W/W' mice. We consider that the total erythropoiesis of these 13 mice was finally reconstituted by a single stem cell with extensive self-maintaining potential.6 In WBB6F1-W/W' mice in which the PGK pattern changed from AB to A or AB to B, the reconstitution appears to result from proliferation of both a single stem cell with extensive self-maintaining potential and stem cell(s) with less self-maintaining potential. The former stem cell probably continued to proliferate after the expiration of the latter stem cell(s).

When bone marrow cells of the reconstituted WBB6F1-W/W' mice in Group A were cultured in methylcellulose medium, both erythroid and nonerythroid colonies with A-type PGK contained macrophages, neutrophils, megakaryocytes, and mast cells. This finding suggests that the hematopoietic stem cells that reconstituted total erythropoiesis of WBB6F1-W/W' mice differentiated into myeloid lineage cells other than erythroid cells. The thymus, lymph node, and Peyer’s patch of the mice in Group A were cultured to produce erythroid and nonerythroid colonies. These results suggest that the stem cells that reconstituted erythropoiesis of the mice differentiate to lymphoid lineage cells. The result is consistent with that of Harrison et al who carried out competitive repopulating analysis after injections of larger numbers of bone marrow cells ($2 \times 10^3 - 2 \times 10^4$).23

A different interpretation, namely the simultaneous use of two different hematopoietic clones, is possible. This interpretation is supported not only by finding B-type PGK in the marrow of mice whose erythrocytes are A-type PGK, but also by finding B-type PGK in erythroid and nonerythroid colonies grown in vitro and derived from the marrow of the same mice.

Reconstitution of only erythrocytes was examined in the erythropoiesis reconstitution assay. When a multipotential hematopoietic stem cell was forced to cure the anemia of a WBB6F1-W/W' mouse, there was a possibility that the stem cell functioned only as an erythroid stem cell and did not repopulate the lymphoid system. However, in this study, we demonstrated that the hematopoietic stem cells that are detected by this reconstitution assay differentiate not only to erythroid cells, but also to other myeloid lineage cells and lymphoid lineage cells. In conclusion, long-term erythropoiesis reconstitution assay is useful for detecting the true primitive hematopoietic stem cells.

ACKNOWLEDGMENT

We thank Dr James N. Ihle of National Cancer Institute for providing Interleukin-3.

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HEMATOPOIETIC STEM CELLS

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