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## Effects of Recombinant Human Granulocyte Colony Stimulating Factor (rhG-CSF) on Hemopoiesis and Survival Rate Following Allogeneic Bone Marrow Transplantation in Mice

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### rhG-CSF のマウス同種骨髄移植後の生存率と 造血能の回復におよぼす影響

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Recombinant Human Granulocyte Colony-Stimulating Factor (rhG-CSF) が同種骨髄移植後の生存率と造血機能の回復におよぼす影響を検討した。無菌動物用ビニールアイソレーター内で飼育している specific pathogen-free C3H マウス（以後 SPF マウス）と無菌 C3H マウス（以後無菌マウス）に対して10Gyの全身照射を行うと9日目までにすべて死亡した。SPF マウスおよび無菌マウスに10Gy全身照射を行い24時間後に specific pathogen-free C57BL マウスの骨髄を利用して同種骨髄移植を行うと生存率は改善されたが、無菌マウスと SPF マウスとでは生存率に差が生じた。無菌マウスでは14日生存率79%（15/19）、100日生存率74%（14/19）に改善されたが、SPF マウスではそれぞれ33%（12/36）、17%（6/36）までにしか改善されなかった。同一の条件下で SPF マウスに対して rhG-CSF を移植翌日より14日間連続投与したところ、移植後5～12日までの死亡が減少し14日生存率79%（30/38）、100日

生存率79%（30/38）と SPF マウスの生存率は無菌マウスと同程度にまで改善された。一方 rhG-CSF 投与による造血機能の回復差を SPF マウスで検討したところ、移植後の生存率を左右すると考えられる移植後10日までの末梢血では、好中球のみが増加していた。すなわち rhG-CSF 投与による生存率の改善は血小板減少や貧血の改善とは考えられなかった。

無菌マウスと SPF マウスの差は消化管の常在菌叢の有無である。全身照射、骨髄移植後の SPF マウスでは内因性の細菌感染、いわゆる日和見感染症が存在するのに対し無菌マウスではこれは存在しない。rhG-CSF の投与は SPF マウスにおける日和見感染症を抑制し生存率を改善したものと考えられた。

同種骨髄移植後に rhG-CSF を投与すると好中球の回復が促進され、日和見感染症が予防され生存率が改善されることが示唆された。

### Summary

The effects of recombinant human granulocyte colony stimulating factor (rhG-CSF) on the survival rate and hemopoiesis after allogeneic bone marrow transplantation (BMT) were examined. Specific pathogen-free (SPF) C3H mice and germ-free C3H mice, kept in an isolator for germ-free animals, received 10 Gy of total body irradiation (TBI) and all the mice died by day 9 after TBI. These survival rates were improved by BMT. In the case of SPF mice, survival rates at 14 and 100 days were 33% (12/36), 17% (6/36) and in the case of germ-free mice they were 79% (15/19), 74% (14/19) respectively. When SPF mice received rhG-CSF (30  $\mu$ g/kg/day) subcutaneously for 14 consecutive days following BMT, their survival rates were improved to 79% (30/38), 79% (30/38) respectively. The survival rate of rhG-CSF treated SPF mice were equal to that of germ-free mice. When the effect of rhG-CSF treatment on hemopoiesis of SPF mice after allogeneic BMT was examined various hematopoietic progenitor cells in the bone marrow and spleen increased until day 10 after, BMT, while only neutrophils increased in the peripheral blood during the period. No adverse effects of rhG-CSF were observed throughout the study period. It was suggested that in SPF mice treated with rhG-CSF after BMT, the neutrophil recovered in counts quickly and increased neutrophil prevented endogenous infections and improved the survival rate without apparent complications.

### Introduction

Bone marrow transplantation (BMT) is expected to become a promising therapy not only for malignant tumors but for aplastic anemia and certain types of congenital metabolic disorders. However, BMT requires conditioning with highly potent immunosuppressive agents and/or total body irradiation (TBI), which lead to myelosuppression. Infections after BMT are sometimes fatal. It has been recently reported<sup>1)-5)</sup> that rhG-CSF improves neutropenia induced by anticancer agents and that it exerts excellent preventive and curative effects on infections. The effect of rhG-CSF on patients after BMT has been also reported<sup>6)-11)</sup>. In the present study, the effects of rhG-CSF on the survival rate after allogeneic BMT and on hemopoiesis were examined serially observing survival rate, changes in the peripheral blood count of the nucleated cells and hematopoietic progenitor cells in the bone marrow and spleen. For the study, germ-free C3H mice and SPF C3H mice were employed. All the mice were housed in a vinyl film isolator for germ-free animals throughout the study period so that exogenous infection would be prevented.

### Materials and Methods

#### Animals

Recipients were SPF C3H/HeN male mice (H-2<sup>k</sup>) which had been purchased at 6 weeks of age from CLEA JAPAN INC. (Tokyo, Japan) and had been kept for 2 weeks in a vinyl film isolator for germ-free animals, and 8-week-old germ-free C3H/HeN male mice (H-2<sup>k</sup>) which had been bred in our laboratory. Donors were SPF C57BL/6N male mice (H-2<sup>b</sup>) which had been purchased at 6 weeks of age from CLEA JAPAN INC. and had been kept for 2 weeks under SPF conditions. The isolator was saturated with 2% per-acetic acid for 2 to 4 hours by spraying it with 0.5 ml per liter volume of the isolator and ventilated more than 8 hours before the study. The incoming air was passed through a HEPA filter. Food and water were placed in sterilizing containers for exclusive use, autoclaved at 121°C for 30 minutes and taken into the isolator through its inlet. A diet for germ-free animals, CL-2 (CLEA JAPAN INC.) was given to the mice. All the host mice were housed in the isolator throughout the study. No antibiotics were administered to the mice.

#### Irradiation

The mice were given 10 Gy of gamma rays (0.47 Gy/minute), using a <sup>60</sup>Co irradiator (Theratron 780,

Atomic Energy of Canada Ltd., Ottawa, Canada).

### **RhG-CSF**

RhG-CSF<sup>12</sup> produced by *E. coli* was provided by Kirin Brewery Co., Ltd., Tokyo, Japan.

### **Survival Rate**

SPF mice and germ-free mice in the isolator were given 10 Gy TBI. After TBI, 12 SPF mice and 14 germ-free mice were kept without any BMT and observed the survival. Nineteen germ-free mice and 74 SPF mice received allogeneic BMT by intracardiac administration of  $1 \times 10^7/0.5$  ml C57BL/6N mouse bone marrow cells at 24 hours after TBI. All the germ-free mice and 36 out of the 74 SPF mice were not further treated after BMT. Twenty-three and 15 out of the 74 SPF mice were treated with rhG-CSF (30  $\mu$ g/kg/day) as a daily single subcutaneous (S.C.) injection for 14 and 21 consecutive days commencing 24 hours after BMT. The survivals of mice were observed for 100 days.

### **Recovery of Hemopoiesis**

One hundred and eighteen SPF C3H mice kept in the isolator received allogeneic BMT under identical conditions to those in the above survival test. The following day, 50 out of the 118 mice were treated with rhG-CSF (30  $\mu$ g/kg/day) as a single S.C. injection for 14 consecutive days. Sixty-four mice out of them were administered the vehicle as a control. The remaining four mice were not treated with rhG-CSF nor the vehicle and were sacrificed on day 0 (the day of BMT). On days 2, 3, 5, 7, 10, 14, and 21 post-BMT, 4 mice from each group were sacrificed for hematological studies. After sacrifice, peripheral blood was taken from the heart, and cell suspension of the bone marrow and spleen were collected.

Peripheral white blood cell (WBC) counts, hemoglobin, hematocrit and platelet count were determined using a Coulter 8/80 (Coulter Electronics Ltd., England). Thin blood films were prepared stained with hematoxylin/eosin and examined microscopically for WBC differential counts.

Bone marrow cells and spleen cells were counted with a Coulter Counter. Hematopoietic progenitor cells in the bone marrow and spleen were determined by culture methods as follows. Bone marrow cells were collected from the femur and tibia using the  $\alpha$ -medium (Flow Laboratories, Inc., Rockville, M.D.), whereas spleen cells were gently ground in a dish containing the  $\alpha$ -medium and collected. The collected bone marrow and spleen cells were suspended in the  $\alpha$ -medium after thorough pipettings. As stimulants, recombinant human erythropoietin (EPO) provided by Kirin Brewery Co., Ltd. and pokeweed mitogen-stimulated murine spleen cell conditioned medium (PWM-CM) were prepared according to the method of Nakahata et al.<sup>13</sup>. The cells were cultured in methylcellulose by modified method of Iscove et al.<sup>14</sup>. A culture medium containing 0.8% methylcellulose (Shinetsu Kagaku Inc., Tokyo, Japan), 30% FCS (Bocknek Laboratories Inc., Toronto, Canada), 1% deionized BSA (Armour Pharmaceutical Co., Kankakee, IL), 2-mercapto-ethanol  $5 \times 10^{-5}$  mol (Eastman Organic Chemistries, Rochester, N.Y.), 10% PWM-CM and 2 IU of EPO was prepared in the Lux culture dishes (#5221R, 35 mm diameter, Miles Laboratories, Inc., Naperville, IL). The bone marrow cells and spleen cells were added to the above medium to make 1 ml of cell culture. The cells were cultured in an incubator at 37°C under 95% air and 5% CO<sub>2</sub> with 100% humidity. Colony types were classified *in situ* with an inverted microscope according to previously reported criteria<sup>13,15</sup>. Colonies were counted on day 6 of culture.

### **Evidence of Bone Marrow Grafts**

The presence of transplanted bone marrow cells was determined in mice of each group that survived for 100 days after allogeneic BMT. The H-2 antigen of spleen cells was analyzed by the Gorer-O'Gorman's conventional cytotoxic assay<sup>16</sup>, using monoclonal antibodies H-2K<sup>k</sup> (Meiji Institute of Health Science, Kanagawa, Japan) and H-2K<sup>b</sup> (Meiji Institute of Health Science). Spleen cells were taken from each mouse, suspended as single cells in medium 199 (Gibco Laboratories, N.Y.), incubated with the H-2 antibodies respectively in water incubator at 37°C for 45 minutes in the presence of complement (LOW-TOXIN-M

RABBIT COMPLEMENT for Use With Mouse Lymphocytes, CL3051, CEDAR LANE LABORATORIES LTD., Ontario, Canada) and stained with 0.16% trypan blue solution. The ratio of surviving cells to dead cells was calculated.

### Statistics

The logrank test was used as a statistical analysis for survival rates and the Student's t-test was used for the laboratory data.

## Results

### 1. Survival Rate

When SPF mice and germ-free mice kept in the isolator were received 10 Gy of TBI, all the SPF mice and all the germ-free mice died 7 to 9 days after TBI respectively (Fig. 1). Germ-free mice and SPF mice received allogeneic BMT at 24 hours after the TBI, their survival rates were improved (Fig. 1); most deaths of the germ-free mice and SPF mice were observed by day 12 post-BMT, from then on deaths were rare. The survival rate of rhG-CSF treated SPF mice improved to the same level as that of germ-free mice (Fig. 1). In case untreated SPF mice, survival rates at 14 and 100 days were 33% (12/36), 17% (6/36) and in case germ-free mice they were 79% (15/19), 74% (14/19) respectively. When SPF mice were treated with rhG-CSF, the survival rates rose to 79% (30/38), 79% (30/38) respectively. Mean survival of the three BMT treated groups were as follows; SPF mice without rhG-CSF treatment was  $30.7 \pm 5.9$  mean  $\pm$  SE) days, germ free mice was  $76.9 \pm 8.9$  days, SPF mice with rhG-CSF was  $81.2 \pm 6.0$  days. The survival of SPF mice was significantly increased by rhG-CSF ( $p < 0.0001$ ). Moreover, the rhG-CSF treated SPF mice did not die after day 12. So there was no difference in survival rate of SPF mice between 14 days-treatment and 21 days-treatment of rhG-CSF.

### 2. Recovery of Hemopoiesis

#### 1) WBC Count

The WBC count in the rhG-CSF group tended to be higher than control group throughout the entire period of observation (Fig. 2-1). The increase of WBC count was statistically significant only at day 10 post-BMT.

#### 2) Neutrophil Count

The neutrophil count in the rhG-CSF group was higher than control group throughout the experiment. For example, the decrease of the neutrophil count after BMT in the rhG-CSF group was milder than control group, in the control group decreased to  $20/\text{mm}^3$ , while in the rhG-CSF group decreased to only  $100/\text{mm}^3$ .

It took 10 days for the neutrophil count to recover to  $1000/\text{mm}^3$  in the control and 7 days in the

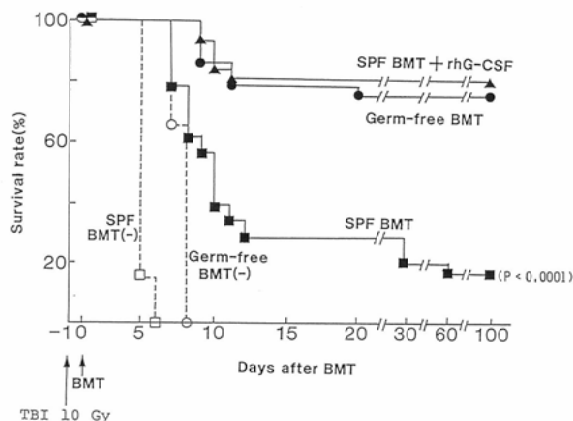


Fig. 1 Survival curves of germ-free and SPF C3H/He mice. SPF BMT (-): SPF mice received 10 Gy of TBI without BMT. Germ free BMT (-): Germ free mice received 10 Gy of TBI without BMT. SPF BMT: SPF mice received TBI and BMT. SPF BMT + rhG-CSF: SPF mice received TBI, BMT and rhG-CSF.

All mice were irradiated 10 Gy of TBI. The day after TBI, three groups of mice were received allogeneic BMT. One group of SPF mice with BMT, was treated by subcutaneous injection of rhG-CSF ( $30 \mu\text{g}/\text{kg}/\text{day}$ ) for 14 consecutive days from the day following BMT. There are significant differences between SPF BMT group and the other BMT treated groups.

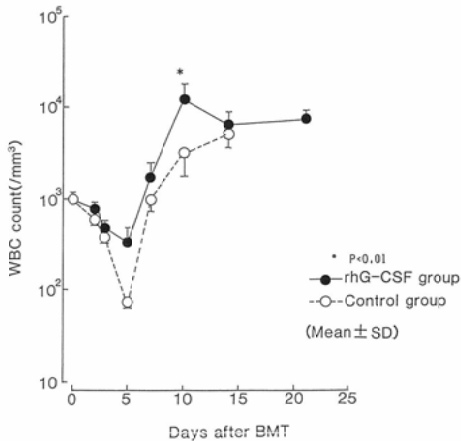


Fig. 2-1 Changes of the WBC count in SPF C3H mice after allogenic BMT. Asterisk marks show the statistically significant difference between two groups.

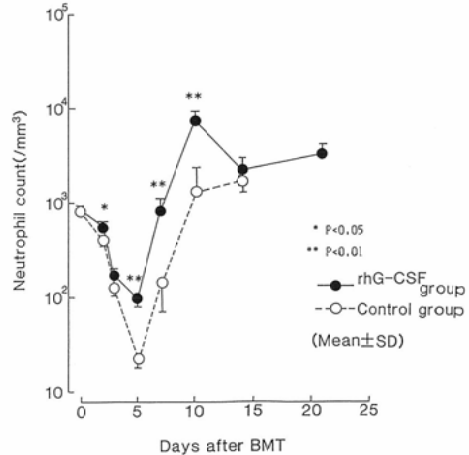


Fig. 2-2 Changes of the neutrophil count in SPF C3H mice after allogenic BMT. Asterisk mark means same as Fig. 2-1.

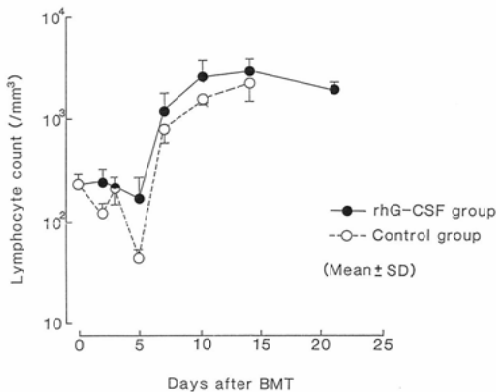


Fig. 2-3 Changes of the lymphocyte count in SPF C3H mice after allogenic BMT.

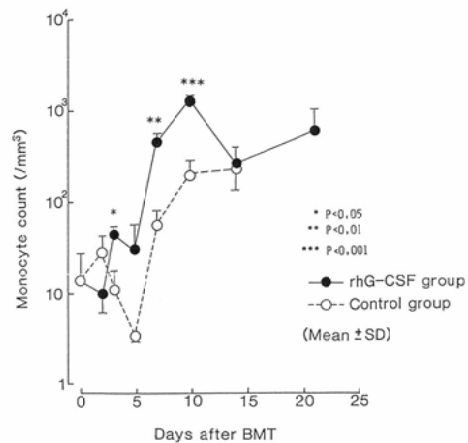


Fig. 2-4 Changes of the monocyte count in SPF C3H mice after allogenic BMT.

rhG-CSF groups respectively, indicating three days shortening of the recovery period. Furthermore, the neutrophil count in the control group never exceeded 2000/mm<sup>3</sup>, whereas that in the rhG-CSF group reached 7000/mm<sup>3</sup> on day 10. The neutrophil count in the rhG-CSF group slightly decreased to 2300/mm<sup>3</sup> on day 14 despite continued rhG-CSF treatment, nevertheless. The neutrophil count after the completion of rhG-CSF treatment (3220/mm<sup>3</sup>) was higher than its normal level. The increases of neutrophil count were statistically significant in the rhG-CSF group at day 2, 5, 7 and 10 post-BMT (Fig. 2-2).

### 3) Lymphocyte Count

No difference in the lymphocyte counts between each group were detected (Fig. 2-3).

### 4) Monocyte Count

The monocyte count in the rhG-CSF group tended to increase earlier than control group. Statistical significance was demonstrated at day 3, 7 and 10 post-BMT (Fig. 2-4).

### 5) Hemoglobin and Hematocrit

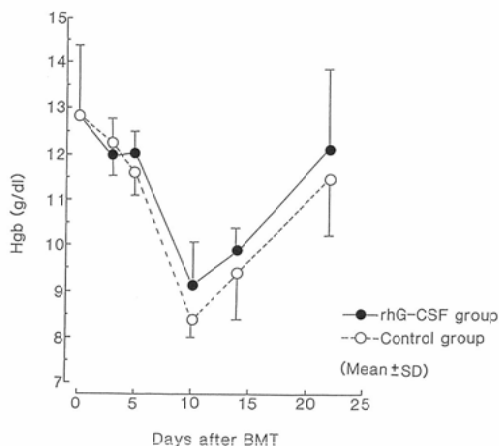


Fig. 3-1 Changes of the hemoglobin in SPF C3H mice after allogeneic BMT.

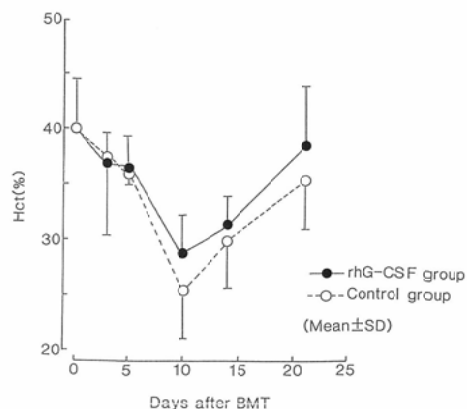


Fig. 3-2 Changes of the hematocrit values in SPF C3H mice after BMT.

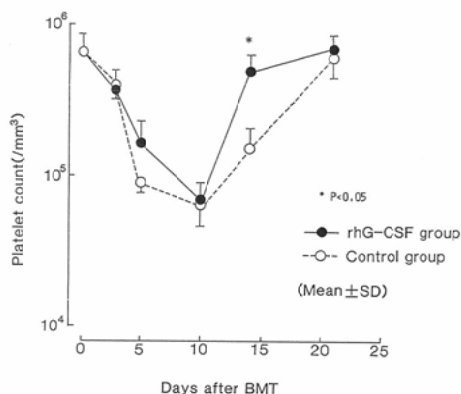


Fig. 4 Changes of the platelet count in SPF C3H mice after allogeneic BMT.

Hemoglobin and hematocrit as expected paralleled each other. Both parameters tended to increase in the rhG-CSF group. But there were not statistically significant (Figs. 3-1, 3-2).

#### 6) Platelet Count

There were no difference in the platelet count except at day 14 post-BMT. The platelet count increased at day 14 post-BMT in rhG-CSF group ( $p < 0.05$ ) (Fig. 4).

#### 7) Nucleated cell Count of the Bone Marrow (femur and tibia) and Spleen

The nucleated cell count in the bone marrow (femur and tibia) and spleen in the rhG-CSF group tended to be higher than control group throughout the study period. The increases of nucleated cell count in the bone marrow were statistically significant at day 2, 3 and 14 post-BMT, and that of spleen at day 10 post-BMT (Figs. 5-1, 5-2).

#### 8) Hematopoietic Progenitor Cells of the Bone Marrow and Spleen

The bone marrow hematopoietic progenitor cell count was not different in the early stage after BMT in the control and rhG-CSF groups. By ten days and later after BMT, that in the rhG-CSF group was higher than control group. There was statistically significant increase at day 10 post-BMT. On the other hand, the

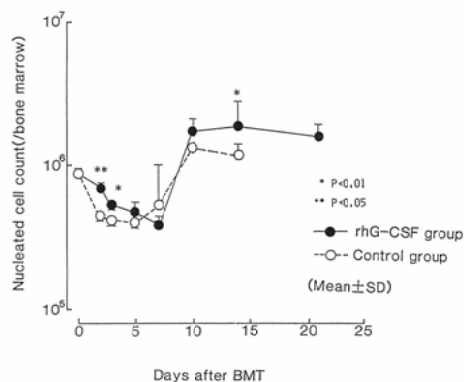


Fig. 5-1 Changes of the nucleated cell count in the bone marrow. Each point shows a mean value of the bone marrow nucleated cell count in the bilateral femur and tibia.

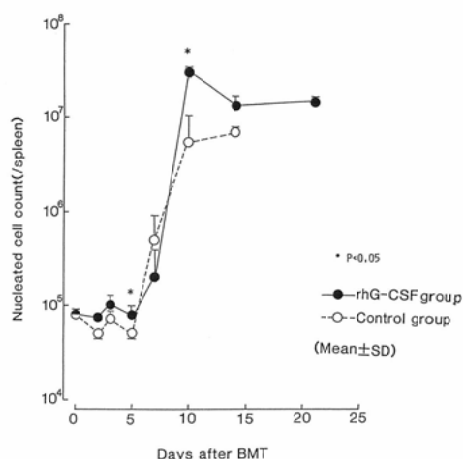


Fig. 5-2 Changes of the nucleated cell count in the spleen.

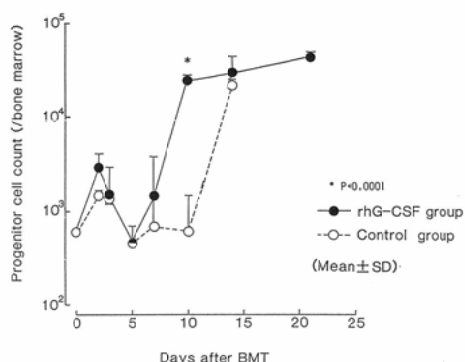


Fig. 6-1 Changes of the hematopoietic progenitor cell count in the bone marrow (femur and tibia).

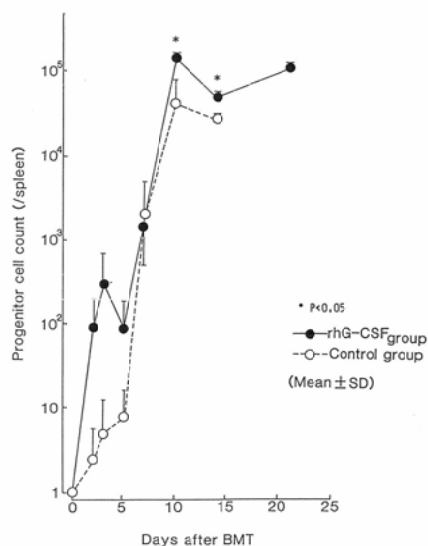


Fig. 6-2 Changes of the hematopoietic progenitor cell count in the spleen.

spleen hematopoietic progenitor cell count increased from the day following BMT until the end of the study period in the rhG-CSF group compared with the controls. Statistical significance was demonstrated at day 10 and 14 post-BMT (Figs. 6-1, 6-2).

### 3. Evidence of Bone Marrow Grafts

The spleen lymphocyte surface antigen of mice that survived for 100 days after BMT was always H-2<sup>b</sup> derived from donor mice (C57BL). H-2<sup>k</sup> antigen derived from host mice (C3H) was not detected. Therefore, donor bone marrow was successfully maintaining hemopoiesis.

### Discussion

Germ-free and SPF mice that had received 10 Gy of TBI were saved by allogeneic BMT. The survival



rate of SPF mice was improved less markedly than that of germ-free mice. It has been reported<sup>17)</sup> that germ-free mice have a higher survival rate than ordinary mice when they are given TBI and treated with allogeneic BMT. Both germ-free mice and SPF mice in the present study were protected from exogenous infections. As SPF mice possessed a normal bacterial flora, it may be that developed endogenous infections during myelosuppression. In contrast the germ-free mice could not develop bacterial infection. This might explain the difference in the survival rate between germ-free and SPF mice. The rhG-CSF treatment to SPF mice after allogeneic BMT improved the survival rate to almost that of the germ-free mice. The above result was attributed to an increase of the survival rate during myelosuppression (on days 5 to 12 post-BMT). The deaths on days 5 to 12 post-BMT might be attributable to hemorrhage due to thrombocytopenia and to infection due to neutropenia. When SPF mice were treated with rhG-CSF after BMT, the peripheral neutrophil count was higher than that of untreated mice. In other words, the neutrophil count in the rhG-CSF group decreased less and then returned earlier to its normal level than the control group. Blood differential counts in the rhG-CSF group on days 5 to 12 showed that only the neutrophil count increased compared with control group. The platelet count was not affected by rhG-CSF treatment by day 10. Accordingly the improvement of survival rate in the rhG-CSF group is not due to a decrease in hemorrhagic deaths since platelet counts were similar during this period. Because rhG-CSF reportedly improves neutropenia seen after radiation or anticancer chemotherapy<sup>1)-11)</sup> and it also rhG-CSF accelerated the recovery of granulocytes, the improvement of survival rate observed in the present study is most likely due to the prevention of infection.

The neutrophil count in the rhG-CSF group increased up to 7000/mm<sup>3</sup> on day 10 but decreased to 2500/mm<sup>3</sup> on day 14 in the present study. On day 21, the neutrophil count in the rhG-CSF group increased slightly again to 3200/mm<sup>3</sup> despite the withdrawal of rhG-CSF treatment. The increase of the neutrophil count on day 10 might be explained by body defense reaction to infection or by transient overreaction during the period of hematopoietic reconstitution.

Additionally hematopoietic progenitor cells in the bone marrow and spleen increased in the early stage after allogeneic BMT in the rhG-CSF group. In the present study, rhG-CSF treatment was started at 24 hours after BMT. Accordingly, rhG-CSF treatment could not have improved the take of transplanted bone marrow cells in the bone marrow and spleen, but the treatment probably improved the differentiation and proliferated bone marrow cells after engraftment in the hematopoietic organs, leading to an increase of hematopoietic stem cells. However, it can not be denied that the recovery of hematopoietic stem cells and peripheral neutrophils in the early stage after BMT might imply the proliferation of hematopoietic progenitor cells remaining in host mouse bone marrow.

In the rhG-CSF group, the hematopoietic progenitor cell count in the bone marrow and spleen were increased, the peripheral monocyte count increased from day 3 and platelet count increased at day 14 respectively with a difference in the time of their increases. This finding suggests that the proliferation of the progenitor cells of both monocytes and megakaryocytes was promoted by rhG-CSF treatment. It was therefore implied that rhG-CSF might stimulate not only neutrophil progenitor cells but also the other immature progenitor cells, as reported previously<sup>18)-20)</sup>.

These results of progenitor cells did not explain the improved survival rate in the rhG-CSF treated group.

The improved survival rate in the rhG-CSF group is most likely due to the prevention of infection on days 5 to 12. It is, therefore, recommended that rhG-CSF treatment should start as early as possible after BMT in order to improve neutropenia during the above period and thus to improve the final survival rate. Accordingly it is concluded that rhG-CSF treatment should be commenced promptly after BMT.

In 14 days-treatment group, no mouse was dead after day 12 post-BMT. In addition, the survival rate of

SPF mice in 21 days-treatment group was as same as that of 14 days-treatment group. This finding may be explained as follows. The neutrophil count in the control group returned to its normal level on day 14, the survival rate in the control group or rhG-CSF group did not change substantially from day 14, bone marrow grafts were established and the neutrophil count was normalized on day 14; thus all the above findings indicate that transplanted mice were out of danger from opportunistic infection on day 14 and later. Therefore, rhG-CSF treatment may be useful when it is conducted before or during myelosuppression after BMT.

As far as the survival rate from day 14 is concerned, three untreated SPF mice died on days 30, 60 and 90 respectively; however, one germ-free mouse died after day 14, and no SPF mice treated with rhG-CSF died after day 14. The cause of death from day 14 was probably due to graft versus host disease (GVHD), because these mice had diarrhea, body weight loss, grayness of the hair and deformity of the tail. So it is not sure whether rhG-CSF treatment may decrease GVHD.

It was shown from the present study that rhG-CSF treatment markedly improved the survival rate of SPF mice receiving allogeneic BMT. Unlike antibiotic therapy, rhG-CSF treatment does not induce adverse effects such as hepatopathy, nephropathy, hearing disorders and myelosuppression. In addition rhG-CSF does not result in appearance of antibiotic resistant bacteria usually observed after extensive antibiotic therapy. Therapy with rhG-CSF promises to become an effective way to prevent infection induced by myelosuppression after BMT, anticancer chemotherapy and radiation therapy.

- 1) RhG-CSF treatment markedly improved the survival rate of SPF mice received allogeneic BMT.
- 2) The survival rate of SPF mice was improved to the same level as that of GF mice.
- 3) Only neutrophils were increased by rhG-CSF during the study period.
- 4) No adverse effects of rhG-CSF were observed through out the period.

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