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Effect of Human Macrophage Colony-stimulating Factor on Granulopoiesis and Survival in Bone-marrow-transplanted Mice

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Human macrophage colony-stimulating factor (hM-CSF) has been isolated from normal human urine and purified to a homogenous protein. The effect of hM-CSF on granulopoiesis was investigated in BALB/c mice transplanted with a suboptimal number of bone marrow cells. Lethally irradiated (7.8 Gy) mice were transplanted with 1×10^6 syngeneic mouse bone marrow cells and treated with a daily intraperitoneal dose of 64 μg/kg of hM-CSF for 5 days following the transplant. The hM-CSF injection resulted in stimulation of the recovery of blood neutrophils as well as an increase in the number of granulocyte-macrophage progenitor cells (CFU-GM) in the femur and spleen. The survival of lethally irradiated mice was dependent on the cell number transplanted; most mice transplanted with 2×10^4 cells died within 2 weeks. The recovery of hematopoiesis in mice transplanted with 2×10^4 cells was modestly but significantly stimulated by hM-CSF administration initiated from 5 days before or 1 day after transplantation for a 5-day period. Furthermore, the hM-CSF administrations markedly reduced the mortality in these mice during the early period after the transplantation. Since anaerobic bacteria were frequently detected in arterial blood immediately before the deaths but were not found in the surviving mice, it is speculated that early deaths occurring within 2 weeks after the transplant may be caused by opportunistic infections, and hM-CSF injection may prevent these mortal infections through its stimulating effect on monocyte-macrophage functions that are responsible for the production of hematopoietic regulators.

Key words: Human macrophage colony-stimulating factor — Bone marrow transplantation — Granulopoiesis — Infection

Most cytotoxic drugs used in cancer chemotherapy injure a variety of hematopoietic progenitor cells as well as malignant cells and frequently cause the development of severe neutropenia in patients.[1)] The neutropenia lowers resistance to infections, and the cancer treatment must be discontinued. Bone marrow transplantation is expected to facilitate reconstitution of hematopoiesis and thus to provide a more effective treatment for patients.[2)] Recently, some of the hematopoietic regulators, such as colony-stimulating factors (CSFs), have been used in conjunction with transplantation in attempts to improve the neutropenia caused by cancer treatment.[3,4)]

Human macrophage colony-stimulating factor (hM-CSF) isolated from normal human urine stimulates the proliferation and differentiation of human monocytic progenitor cells (CFU-M) as well as the function of mature monocyte-macrophage cells.[5)] The hM-CSF has also been applied in the treatment of neutropenia caused by cancer chemotherapy and has been reported to improve granulocyte production in these patients.[6)] It is believed that monocyte-macrophages are a major cell source of granulopoietic growth factors [e.g., granulocyte CSF (G-CSF) and granulocyte-macrophage CSF (GM-CSF)] and may be strongly related to the resistance to infections. The stimulating effect of hM-CSF on granulocyte recovery might be promoted through its enhancing effect on the G-CSF and GM-CSF production by macrophages.

In this study, we investigated the effect of hM-CSF on the neutropenia and infections that occur at an early period in bone-marrow-transplanted mice.

MATERIALS AND METHODS

Mice Specific pathogen-free male mice of inbred BALB/c strain at 6 to 8 weeks of age were purchased from Charles River Japan Inc. (Atsugi, Kanagawa) and acclimated to conventional conditions for 2 weeks. Standard mouse chow (Oriental Yeast Manuf. Co., Tokyo) and sterilized water were given *ad libitum*. After the

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radiation and the bone marrow transplantation, they were placed in a clean isolator (TAR-40, Toyo Riko Co., Tokyo) and fed under aseptic conditions with circulation of filtered air.

Bone marrow transplantation Then mice accommodated in a cage covered with a sterile filter cap (Japan Clea Co., Tokyo) were irradiated at 0.13 Gy/min for 1 h with a ^{60}Co source (total dose of 7.8 Gy) in a shielded room at the Tokyo Metropolitan Institute of Isotopes (Meguro, Tokyo). At 2 h after irradiation, a 0.2 ml aliquot of bone marrow cells obtained from normal syngeneic mice and suspended in Dulbecco's buffered saline (DBS, Flow Laboratories, Irvine, UK) was infused intravenously at cell numbers of 10^7, 10^6, 10^5, or 2×10^4 via the tail vein.

Preparation and administration of hM-CSF The hM-CSF was purified from normal human urine by a 6-step purification procedure involving a reversed-phase high-performance liquid chromatography (RP-HPLC) as a final purification step.[7] The purified material revealed a single band having an apparent molecular weight of 85 kDa on non-reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis.[8] Biological activity was assayed by the standard mouse bone marrow system,[9] and one unit was defined as the amount that was needed to form a mouse monocytic colony in the agar gel. The specific activity of purified hM-CSF used in this study was 1×10^8 units/mg protein. To avoid adherence of hM-CSF to plastic vessels, the purified material was dissolved at 6.4 μg/ml in Dulbecco's buffered saline containing 0.001% human serum albumin (Green Cross Corporation, Osaka). The hM-CSF preparation and vehicle contained no lipopolysaccharide as determined by the *Limulus* amebocyte lysate assay (less than 0.06 EU/ml; Whittaker Bioproducts, Walkersville, MD). The administration dose of hM-CSF was determined in normal mice. Our preliminary study demonstrated that intraperitoneal (ip) injection of hM-CSF at a daily dose of 32 to 64 μg/kg for a 5-day period induced the maximum increase in number of CFU-M in both the femur and spleen in a dose-dependent manner. Thus, ip injection of hM-CSF at a daily dose of 64 μg/kg for a 5-day period was used in this study. To assess the granulopoietic effect of hM-CSF on the bone-marrow-transplanted mice, the hM-CSF administration was performed in lethally irradiated mice transplanted with 10^6 or 2×10^4 syngeneic bone marrow cells. In the case of 10^6 cells, daily administration of hM-CSF was initiated from 1 day after the engraftment and continued for a 5-day period. In another case of 2×10^4 cells, the hM-CSF injection was started at 5 days before (−Day 5), and 1 day (Day 1) or 5 days after (Day 5) the bone marrow transplantation and continued for a 5-day period. The control group was given the vehicle from 1 day after the engraftment for a 5-day period. In order to ascertain the protective effect of hM-CSF on the mortality of mice transplanted with 2×10^4 cells, the administrations of −Day 5 and Day 1 were examined in the survival experiment by using 10 mice per injection group. The survival experiment was repeated twice to confirm the reproducibility of the effect of hM-CSF on the survival.

Quantity of progenitor cells and peripheral blood cells Four to 5 mice per group were killed at various time intervals, and femoral shaft and spleen were aseptically removed from each mouse. The number of progenitor cells in the femur and spleen was measured by a soft agar culture method as previously reported.[9] Total myeloid progenitor cells (CFU-GM) were assayed by using a pokeweed-mitogen-stimulated BALB/c mouse spleen cell conditioned medium (PWM-SCM) and monocyte-macrophage progenitor cells (CFU-M) were assayed by using hM-CSF as a stimulator. Peripheral blood samples were taken from the tail vein. The nucleated cells obtained from both femur and spleen and the peripheral blood cells were counted by an automatic cell counter (TOA CC-180A, Toa Medical Electronics, Kobe). Differential counting of leukocytes was performed on a smeared sample stained with Wright-Giemsa's solution (Merck, Darmstadt, West Germany).

Cultivation of the arterial blood samples To survey whether microbial infections occurred in mice transplanted with a small number of bone marrow cells (2×10^4), the cultivation of arterial blood was performed to detect the presence of bacteria in the circulating blood. The arterial blood samples were obtained by cardiac puncture under ether anesthesia from mice immediately before death and from surviving mice, and 200 μl of each blood sample was inoculated into a tryptosoy-thioglycolate broth (Difco Laboratories, Detroit, MI) and incubated at 37°C for 7 days.

Statistical analysis The two-tailed Student's t test and χ^2 test were used for statistical evaluation of the data from the hematopoietic and survival experiments.

RESULTS

Effect of hM-CSF on the hematopoiesis in bone-marrow-transplanted mice The survival and hematopoietic reconstitution of lethally irradiated mice were dependent on the amount of bone marrow cells engrafted. When 10^7 bone marrow cells were transplanted, the hematopoietic functions rapidly recovered within 2 weeks. Mice transplanted with 10^6 cells did not die; however, the recovery of peripheral leukocyte and thrombocyte counts was delayed for at least 2 weeks. On the other hand, restoration of hematopoiesis in mice transplanted with 10^5 cells was not observed and half of the mice died within 2 weeks (data not shown).

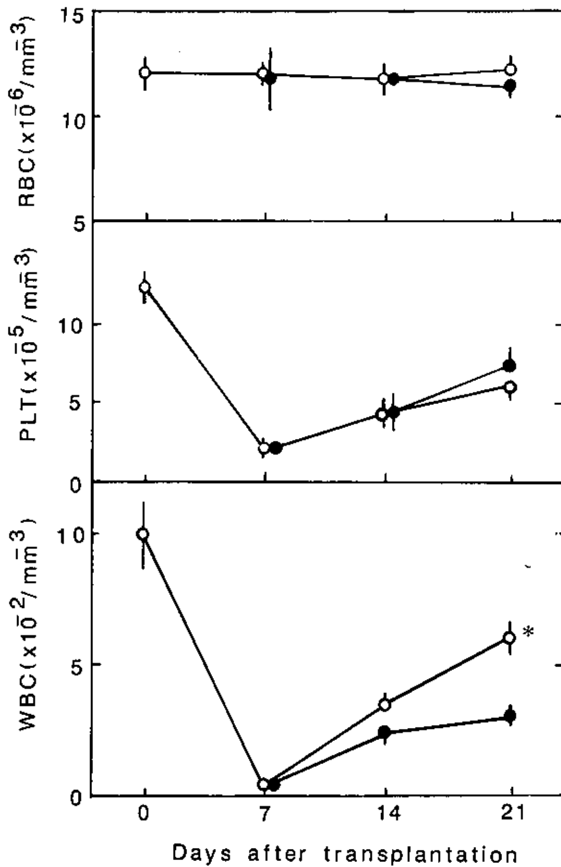

Fig. 1. Recovery of peripheral blood cells upon hM-CSF injection in bone-marrow-transplanted mice. After lethal irradiation, mice were transplanted witn 10^6 bone marrow cells and daily given 64 μg/kg of hM-CSF (○) or vehicle (●) for a 5-day period from the next day after the engraftment. Symbols represent the mean ± SD. The criterion of statistical significance was $P<0.05$ (*).

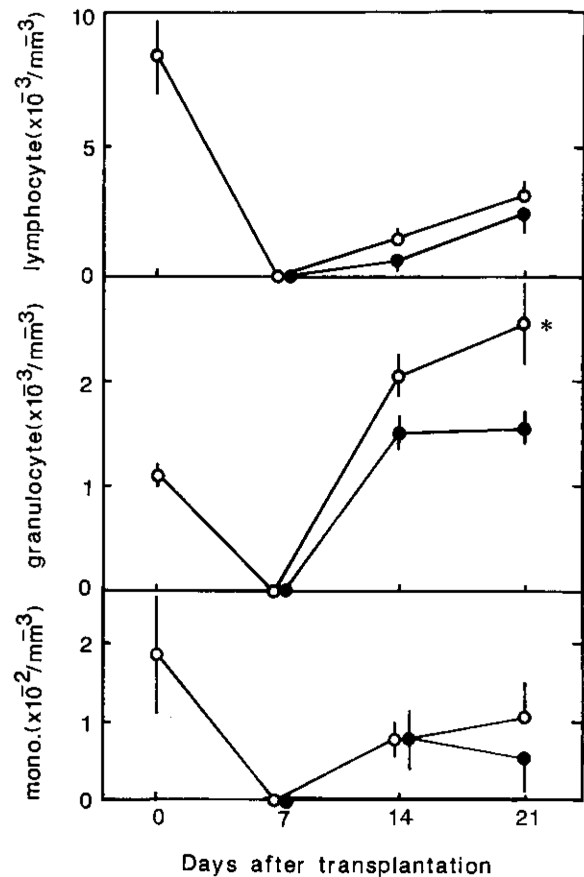

Fig. 2. Changes of differential leukocyte counts by hM-CSF injection in bone-marrow-transplanted mice. A statistically significant difference between the hM-CSF (○) and vehicle (●) groups was found with $P<0.05$ (*).

To evaluate the hematopoietic effect of hM-CSF on bone marrow transplantation, 5 consecutive injections of hM-CSF were performed on mice engrafted with 10^6 cells and the data was compared with those for control mice injected with the vehicle. The bone-marrow-transplanted mice revealed marked leukocytopenia and thrombocytopenia at 7 days after irradiation and gradual recovery was observed between day 14 and day 21 (Fig. 1). The regeneration of peripheral leukocytes between day 14 and day 21 was obviously stimulated by the repeated injections of hM-CSF ($P<0.05$) and the increase in leukocyte counts was due to the increase in number of neutrophilic granulocytes (Fig. 2). In the fluctuation of hematopoietic cells, marked proliferation of CFU-GM was observed in spleen rather than in bone marrow. The increases in CFU-GM numbers as well as in CFU-M numbers in both organs were effectively enhance by hM-CSF administration ($P<0.05$, Fig. 3).

Lethally irradiated mice transplanted subsequently with a small number of bone marrow cells (2×10^4) died during the early period after the engraftment when they were injected with vehicle. The hM-CSF injections were performed for a 5-day period starting at 5 days before (−Day 5) and 1 day (Day 1) or 5 days (Day 5) after the bone marrow transplantation. The changes in peripheral blood cells and in progenitor cells in both femoral marrow and spleen are presented in Tables I and II. Since more than half of the bone-marrow-transplanted mice died during the experimental period, statistical evaluation of the data could not be carried out. Although serious leukocytopenia still remained and the numbers of nucleated cells and CFU-GM in femoral marrow were

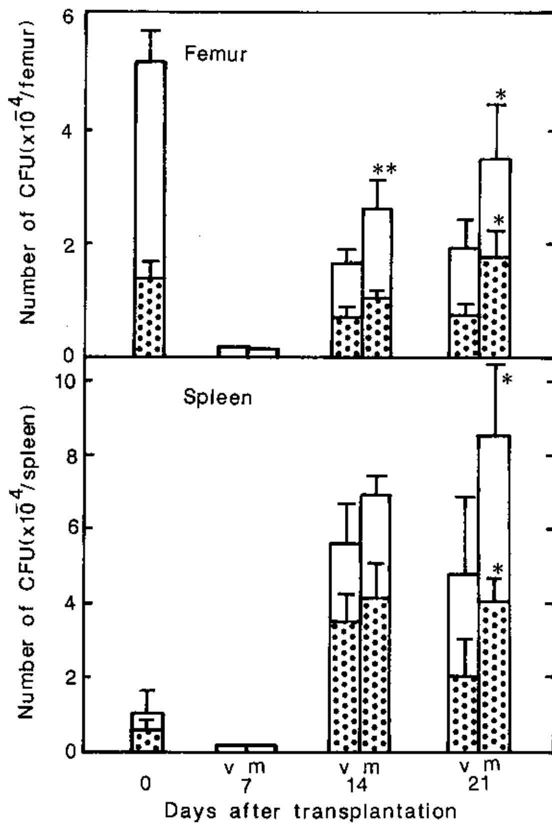

Fig. 3. Increases in numbers of total myeloid progenitor cells (CFU-GM, □) and monocyte-macrophage progenitor cells (CFU-M, ⊡) by hM-CSF injection. Statistically significant differences between hM-CSF-injected (m) and vehicle-injected (v) groups were found with $P<0.05$ (*) or $P<0.01$ (**).

extremely low, it was observed that the splenic CFU-GM numbers in the −Day 5 and the Day 1 groups were obviously increased, and the erythrocyte counts at day 14 in both groups were higher than that of the control group. In addition, the numbers of surviving mice at day 21 in both the −Day 5 and Day 1 groups were higher than those of the control and Day 5 groups (Table I). On the other hand, the effect of Day 5 injection on the peripheral leukocytes and splenic CFU-GM number was similar to those of −Day 5 and Day 1 injections; however, it had no reducing effect on the mortality.

The effect of hM-CSF on the survival of mice transplanted with a small number of bone marrow cells In order to ascertain the effect of hM-CSF on the survival of mice transplanted with 2×10^4 cells, −Day 5 and Day 1 injections of hM-CSF were further investigated in two experiments using 10 mice per group. In experiment 1 (Fig. 4), hM-CSF administrations could not prevent death in mice transplanted with a suboptimal number of bone marrow cells; however, early death occurring within 2 weeks after the bone marrow transplantation was remarkably reduced and the survival periods were prolonged by hM-CSF injections. Comparing the survival rates of each group at day 14, those of the −Day 5 and Day 1 groups were 90%, whereas those of the two control groups (vehicle and no-BMT groups) were 20% and 10%, respectively. In experiment 2, although the surviving mice in both the −Day 5 and Day 1 groups were killed to obtain blood samples at days 14 and 21, the survival rates of hM-CSF-injected groups at day 14 were also more than 80%, whereas that of the control group was only 30%. In contrast to experiment 1, no death was observed in the −Day 5 and Day 1 groups during the period from day 14 through day 21. As shown in Table III, anaerobic bacteria were frequently detected in the blood samples of dead mice, but not in those of surviving mice, suggesting that early death occurring within 2 weeks after the lethal irradiation was caused by opportunistic infections.

DISCUSSION

The aim of hM-CSF administration in neutropenia caused by chemotherapy and irradiation for bone marrow transplantation is to stimulate the regeneration of normal granulopoiesis in the patients. On the basis of neutrophil production *in vivo,* the granulopoietic regulators, G-CSF or GM-CSF, act on the myeloid hematopoietic progenitor cells (CFU-GM) and stimulate their proliferation and differentiation to produce mature neutrophilic granulocytes.[10, 11] Previous authors have reported marked granulocytosis in neutropenic patients[12, 13] and animals[14, 15] when they were injected with an appropriate dose of G-CSF or GM-CSF. Furthermore, peripheral neutrophilia is observed subsequently to elevation of serum CSF activity in the infected animals.[16] The monocyte-macrophage lineage cells[17] and T-lymphocytes[18] are major cell sources of G-CSF and GM-CSF and it is believed that they produce these regulators in response to infections and/or certain hematopoietic destruction caused by chemotherapy and irradiation. In contrast to G-CSF and GM-CSF administration, it has been indicated that hM-CSF stimulated the G-CSF and GM-CSF production by human monocytes *in vitro*[5] and the serum elevation of G-CSF activity in neutropenic patients.[19] These findings suggest that the granulopoietic effect of hM-CSF might be evoked through the stimulating action on endogenous productions of G-CSF and GM-CSF by monocyte-macrophage cells.

In this study, we have investigated the stimulating effect of hM-CSF on the regeneration of granulopoiesis in bone-marrow-transplanted mice. Although the hematopoietic functions lethally irradiated mice are completely

Table I. Effect of hM-CSF Administrations on Peripheral Blood Cell Counts and Mortality in Mice Transplanted with 2×10^4 Bone Marrow Cells

Groups	Days after transplant.	RBC ($\times10^{-4}$)	PLT ($\times10^{-4}$)	WBC ($\times10^{-2}$)	Mortality[a]
−Day 5	7 (n=4)	1,107±20	18±6	5±2	
	14 (n=3)	929±60	2±1	6±1	
	21 (n=4)	665±237	12±2	28±8	0/4
Day 1	7 (n=4)	1,028±24	14±2	4±0	
	14 (n=3)	828±45	4±2	4±2	
	21 (n=3)	817±44	14±2	26±4	1/4
Day 5	7 (n=4)	1,110±14	15±1	6±1	
	14 (n=2)	779±96	6±1	4±1	
	21 (n=0)	—	—	—	4/4
Control	7 (n=4)	1,148±27	19±4	6±2	
	14 (n=3)	729±56	3±1	6±1	
	21 (n=1)	1,010	10	15	3/4
No BMT[b]	4 (n=4)	1,134±19	84±9	3±1	
	14 (n=3)	472±78	2±0	4±1	
	21 (n=0)	—	—	—	4/4
Normal	(n=4)	1,194±53	136±1	81±5	

a) Number of deaths per cage at day 21.
b) Mice were irradiated at the same dose as other groups and were not transplanted with bone marrow cells.
Each value represents the mean±SD. The control group did not receive hM-CSF.

Table II. Comparisons of Myeloid Progenitor Cell (CFU-GM) and Nucleated Cell Numbers in Bone Marrow and Spleen at 14 Days after the Bone Marrow Transplantation

Groups	Bone marrow		Spleen	
	nucleated cells ($\times10^{-6}$/femur)	CFU-GM (per femur)	nucleated cells ($\times10^{-6}$/spleen)	CFU-GM ($\times10^{-2}$/spleen)
−Day 5	1.7±0.6	8±6	18.2±8.6	81.4±93.7
Day 1	2.8±0.4	287±203	11.9±5.6	100.3±127.4
Day 5	2.3±0.4	170±123	6.6±2.0	75.2±71.8
Control	2.4±0.4	157±124	5.0±2.2	6.0±3.9
No BMT	2.3±0.1	0	0.3±0.2	0.1±0.1
Normal	22.3±2.1	49,991±5,286	133.7±21.1	95.7±26.2

After lethal irradiation, mice were engrafted with 2×10^{-4} bone marrow cells. Each value represents the mean±SD of 3 mice. The control group was administered with vehicle and the no-BMT group received neither bone marrow transplantation nor hM-CSF injection.

destroyed, the reconstruction of normal hematopoiesis could be afforded by transplantation of syngeneic mouse bone marrow cells. The recovery of peripheral blood cells is dependent on the number of cells transplanted and a rapid increase in the number of leukocytes is observed when a sufficient number of bone marrow cells containing the hematopoietic progenitor cells and the growth factor-producing cells is infused. However, when an inadequate number of bone marrow cells is transplanted, hematopoiesis, especially granulopoiesis, does not occur and most of the mice die in the early period after the lethal irradiation.

The repeated injections of hM-CSF obviously stimulated the granulopoiesis in mice transplanted with a suboptimal number of bone marrow cells. The CFU-GM newly introduced by bone marrow transplantation pro-

liferated in the spleen rather than in bone marrow, and hM-CSF stimulated the increase in the number of CFU-GM as well as the CFU-M in both organs. Consistent with the marked recovery of CFU-GM, peripheral neutrophils were also increased by hM-CSF injections, while monocyte and thrombocyte counts were not significantly affected. On the other hand, marked changes in the peripheral blood cells were not apparent in mice transplanted with an inadequate number of bone marrow cells even when they were given hM-CSF injections; however, early death occurring within 2 weeks after the irradiation was significantly reduced and the survival period was prolonged by the hM-CSF injections. In particular, the pretreatment with hM-CSF, starting at 5 days before the irradiation, seemed to be more effective than the post-treatment with hM-CSF initiated from 1 day after the transplantation. Since the arterial blood samples obtained from mice just before death frequently contained anaerobic bacteria, it is speculated that death occurring in the early and severe neutropenic period might be due to opportunistic infections and the hM-CSF administration may temporarily prevent these mortal infections. Because the protective effect of antibiotics on bacterial infections is often reduced in severe neutropenia, it is thought that hM-CSF administration should be very useful to treat the neutropenia caused by irradiation for bone marrow transplantation.

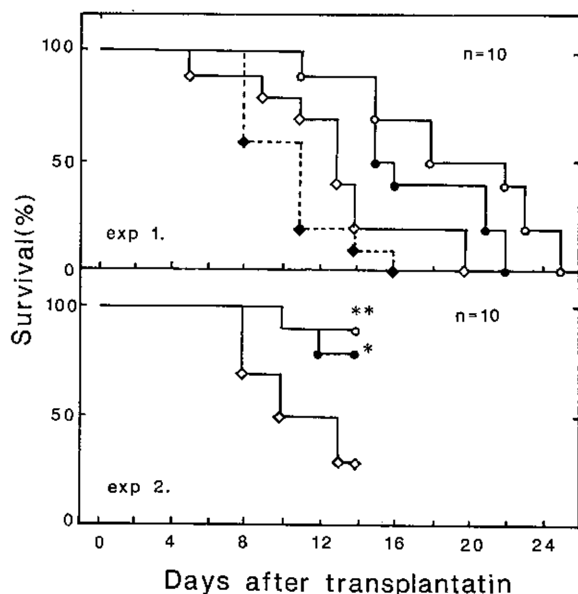

Fig. 4. Effect of hM-CSF administration on the survival of mice engrafted with a small number of bone marrow cells. Ten mice per group were used in these experiments. The hM-CSF administrations were performed at 5 days before (○) or 1 day after (●) the transplantation for a 5-day period. The control group was given the vehicle (◇) from 1 day after the transplantation. In experiment 1, lethally irradiated mice without bone marrow transplantation were used as another control group (◆). In experiment 2, because the surviving mice in both hM-CSF-injected groups were killed at days 14 and 21, the complete survival periods was not obtained. Statistically significant differences in survival ratios at day 14 were found against the vehicle group with $P<0.05$ (*) or $P<0.01$ (**).

As described above, it is also suggested that hM-CSF modulates the granulopoiesis in bone marrow-transplanted mice as well as in neutropenic patients and animals treated with cytotoxic drugs through its enhancing effect on the cytokine production by monocyte-macrophages. Consistent with this speculation, we have confirmed that hM-CSF could stimulate the interleukin-1 (IL-1) production by mouse peritoneal macrophages *in vitro* and the elevation of serum CSF activity in leukocytopenic mice treated with cyclophosphamide (manuscript submitted for publication). IL-1 is another potential cytokine produced by monocyte-macrophages and plays a role as a modulator in hematopoiesis and immunological reactions.[20, 21] Neta and coworkers[22]

Table III. Examination of Bacteria in Arterial Blood Samples Obtained from Mice Transplanted with 2×10^4 Bone Marrow Cells

Groups	Death			Survival		
	No. of deaths	No. examined	No. positive	No. of survivals	No. examined	No. positive
Control	7	5	3	3	3 (day 21)	0
−Day 5	1	1	1	9	4 (day 14)	0
					5 (day 21)	0
Day 1	2	1	1	8	4 (day 14)	0
					4 (day 21)	0

Human M-CSF administrations were initiated from 5 days before or 1 day after the bone marrow transplantation. Control group was injected with vehicle from day 1.

have demonstrated that pretreatment with IL-1 reduced the mortality in lethally irradiated mice. The radioprotective roles of IL-1 have not yet been elucidated; however, it stimulates the release of G-CSF and GM-CSF from endothelial cells, which, like macrophages, are another one of the major cell sources of these regulators and they participate as a component of the hematopoietic microenvironment in stroma.[23] At present, it is still not clear whether hM-CSF enhances the IL-1 production as well as G-CSF and GM-CSF productions by macrophages in bone-marrow-transplanted mice. Further study on the cytokine production networks should be carried out in bone-marrow-transplanted mice.

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