In Vitro Proliferation of Primitive Hemopoietic Stem Cells Supported by Stromal Cells: Evidence for the Presence of a Mechanism(s) Other Than That Involving *c-kit* Receptor and Its Ligand

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Summary

The preadipose cell line, PA6, can support long-term hemopoiesis. Frequency of the hemopoietic stem cells capable of sustaining hemopoiesis in cocultures of bone marrow cells and PA6 cells for 6 wk was $1/5.3 \times 10^4$ bone marrow cells. In the group of dishes into which bone marrow cells had been inoculated at 2.5×10^4 cells/dish, 3 of 19 dishes (16%) contained stem cells capable of reconstituting erythropoiesis of WBB6F$_1$-*W/W^v* mice, indicating that PA6 cells can support the proliferation of primitive hemopoietic stem cells. When the cocultures were treated with an antagonistic anti-c-*kit* monoclonal antibody, ACK2, only a small number of day 12 spleen colony-forming units survived; and hemopoiesis was severely reduced. However, when the cocultures were continued with antibody-free medium, hemopoiesis dramatically recovered. To examine the proliferative properties of the ACK2-resistant stem cells, we developed a colony assay system by modifying our coculture system. Sequential observations of the development of individual colonies and their disappearance demonstrated that the stem cells having higher proliferative capacity preferentially survive the ACK2 treatment. Furthermore, cells of subclones of the PA6 clone that were incapable of supporting long-term hemopoiesis expressed mRNA for the c-*kit* ligand. These results suggest that a mechanism(s) other than that involving c-*kit* receptor and its ligand plays an important role in the survival and proliferation of primitive hemopoietic stem cells.

The hemopoietic stem cell is characterized by its extensive self-maintenance capacity and differentiation potential (1). It is well established that a single stem cell can give rise to cells of all lymphohemopoietic lineages (2–4). Such stem cells have been operationally defined and assayed by their capacity for long-term repopulation of lymphohemopoietic cells after injection of them into either lethally irradiated or genetically anemic WBB6F$_1$-*W/W^v* mice (5–8). These stem cells are physically separable from the majority of the cells that form macroscopic colonies in the spleens of lethally irradiated mice (spleen colony-forming units [CFU-S][1]) (9–11).

However, biological properties of the primitive hemopoietic stem cells are poorly understood. One of the difficulties in studying this problem is the absence of an in vitro assay system of the stem cells. In the long-term bone marrow culture system, hemopoiesis is sustained for several months in close association with an adherent stromal cell layer (12). Although primitive hemopoietic stem cells are known to be present in this culture system for at least 4 wk (13–15), neither have they been quantitatively assessed nor has the mechanism responsible for their proliferation and differentiation been clarified.

On the other hand, mice bearing mutations at the dominant white spotting (*W*) locus have long been believed to have a defect intrinsic to hemopoietic stem cells (16). The *W* locus has been shown to be allelic with the c-*kit* proto-

[1] *Abbreviations used in this paper:* CFU-S, spleen colony-forming units; DEX, dexamethasone; HS, horse serum; *Sl*, steel; *W*, white spotting.

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oncogene, a member of the transmembrane tyrosine kinase receptor family (17, 18). Furthermore, the ligand for this receptor (c-*kit* ligand) has been identified as the product of the steel (*Sl*) locus (19–21).

Recently, a mAb, ACK2, recognizing an extracellular domain of the c-*kit* receptor molecule has been developed (22, 23). This antibody has been strongly suggested to antagonistically block the function of the c-*kit* receptor and to be cytostatic rather than cytotoxic for the cells expressing its antigen (22, 23). Most hemopoietic progenitor cells, including the stem cells capable of reconstituting in vivo lymphohemopoiesis, have been shown to express the c-*kit* molecule (23–25). Most of the clonogenically assayable progenitor cells in bone marrow are depleted by the injection of ACK2 (23). Also, colony formation of CFU-S is inhibited by the injection of the antibody (24). These findings demonstrate that the c-*kit* molecule and its ligand play an essential role in the constitutive hemopoiesis in vivo. However, a small but significant fraction of day 13 CFU-S is resistant to the antagonistic antibody (23), and sensitivity of day 12 CFU-S to ACK2 is significantly lower than that of day 8 CFU-S (24). These findings raise the question as to whether the c-*kit* receptor is functionally requisite for the proliferation of the hemopoietic stem cells at an early stage.

In this study, we found that our preadipose cell line, MC3T3-G2/PA6 (PA6) (26–28), can support the proliferation of hemopoietic stem cells capable of reconstituting whole erythropoiesis of WBB6F$_1$-*W/W^v* mice for 24 wk. By modifying the coculture system of bone marrow cells and PA6 cells, we developed an in vitro colony assay system for the detection of hemopoietic stem cells having varying proliferative capacities. Then, taking advantage of the ACK2 mAb and subclones of PA6 clone that were incapable of supporting long-term hemopoiesis, we obtained data strongly suggesting the presence of a mechanism(s) other than that involving the c-*kit* molecule and its ligand in the survival and proliferation of primitive hemopoietic stem cells.

Materials and Methods

Mice. C57BL/6CrSlc, WBB6F$_1$-*W/W^v*, and WB/Re mice were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). These mice were used at 6–10 wk of age.

Cell Lines. All cultures were incubated at 37°C in a fully humidified atmosphere of 5% CO_2 in air. The clonal preadipose cell line PA6 (26–28) and the bone marrow–derived stromal cell line ST2 (29, 30) have been described previously. Subclones 2, 12, and 14 were isolated from the parental PA6 clone and selected on the basis of their inability to support long-term hemopoiesis.

Anti-c-kit mAb and Recombinant Mouse c-kit Ligand (rmc-kit Ligand). Purified ACK2 mAb recognizing an extracellular domain of the mouse c-*kit* molecule was prepared as described previously (22, 23). For the preparation of rmc-*kit* ligand, cDNA corresponding to the extracellular domain of mouse c-*kit* ligand (amino acids 1–185) was amplified by the reverse transcription (RT)-PCR method and cloned into pYZ10 yeast expression vector. The vector was transfected into yeast to result in the secretion of rmc-*kit* ligand into culture medium. The culture medium was concentrated by ultrafiltration and subjected to partial purification steps by Mono Q anion-exchange (Pharmacia LKB Biotechnology, Uppsala, Sweden) and Phenyl-superose (Pharmacia LKB Biotechnology) column chromatography. Activity of rmc-*kit* ligand was detected by the inhibition assay of ACK2 binding to IL-3-dependent mast cells derived from normal mice. Active fractions were pooled, concentrated, and dialyzed against PBS$^-$. Concentration of rmc-*kit* ligand was estimated by SDS-PAGE, and working dilution of this preparation was determined by the colony assay of mouse bone marrow cells.

Coculture of Bone Marrow Cells with PA6 Cells. Confluent cell layers of parental PA6 clone, one of its subclones, or a 1:1 mixture of PA6 clone and one of the subclones were established as reported previously (28) in 35-mm dishes (Sumitomo Bakelite, Tokyo, Japan) coated with type I collagen (Nitta Gelatin, Osaka, Japan). Bone marrow cells from femurs of female C57BL/6 mice were inoculated onto the preadipocyte layers at 3×10^5 cells/dish and cocultured with 1.5 ml of α-MEM (Irvine Scientific, Santa Ana, CA) supplemented with 20% horse serum (HS; HyClone Laboratories, Logan, UT) and 10^{-8} M dexamethasone (DEX). Medium was changed twice a week. ACK2 antibody was added to the cocultures at the concentration of 0.1, 1, or 10 μg/ml. After 4 or 14 d of the antibody treatment, cocultures were washed three times with medium and then continued with antibody-free medium. For the pretreatment of bone marrow cells with the ACK2, bone marrow cells were suspended at 2×10^5 cells/ml in the medium containing 10 μg/ml ACK2 and incubated at 37°C for 1 h. rmc-*kit* ligand was added at the concentration of 50 ng/ml. At every twice-a-week medium change, nonadherent hemopoietic cells were harvested by gentle pipetting and rinsing once with medium and pooled. Number of the cells was determined with a hemocytometer. CFU-S assay was performed by counting of spleen colonies at 12 d after injection as described previously (27, 28).

In Vitro Limiting Dilution Assay and In Vivo Reconstitution Assay of Hemopoietic Stem Cells. Bone marrow cells were inoculated onto the cell layers of parental PA6 clone at varying cell dilutions (6.25×10^3 to 1×10^5 cells/35-mm dish) and cocultured as described above. On days 28, 35, and 42 of coculture, nonadherent cells were harvested and counted as described above. Dishes containing $>5 \times 10^5$ nonadherent cells were judged as hemopoiesis positive. Each cell dilution consisted of 15–20 dishes. Frequency of the hemopoietic stem cells capable of sustaining hemopoiesis within the cocultures in the bone marrow cell population was calculated according to the method described by Porter and Berry (31), Brevik (32), and Boggs et al. (5).

On day 42, from the hemopoiesis-positive dishes in the group in which bone marrow cells had been inoculated at 2.5×10^4 cells/dish, hemopoietic cells associated with the adherent cell layers were harvested by treatment with 0.1% collagenase (Nitta Gelatin) and removal of adherent cells by 1-h adherence to a plastic surface. The hemopoietic cells from individual dishes were intravenously injected into each WBB6F$_1$-*W/W^v* mouse. At 8, 12, 16, 20, and 24 wk after the injection, peripheral blood of the mice was obtained by retroorbital puncture under anesthesia. Electrophoretic pattern of hemoglobin was determined after modification with cystamine according to the method described by Whitney (33). Hemoglobin of donor C57BL/6 mice carrying the "single" allele (*Hbb^s/Hbb^s*) can be distinguished from that of recipient WBB6F$_1$-*W/W^v* mice carrying heterozygous combination of "diffuse" and "single" alleles (*Hbb^d/Hbb^s*).

In Vitro Colony Assay of Hemopoietic Stem Cells. Bone marrow cells were inoculated onto the cell layers of parental PA6 clone at 5×10^4 or 2×10^5 cells/60-mm dish (Sumitomo Bakelite) and cocultured in the absence or presence of 10 μg/ml ACK2 as described above. On day 2 or 4 of coculture, after having been washed

three times with medium, adherent cell layers were covered with 2 ml of medium consisting of α-MEM, 0.08% type I collagen, 20% HS, and 10^{-8} M DEX. After the collagen had been allowed to gel at room temperature for 20 min, the cocultures were resumed. The next day, 3 ml of medium was added onto the gel. Then the medium was changed twice a week. Twice a week, colonies having a diameter of 2 mm or more and containing immature hemopoietic cells and/or both neutrophils and macrophages were counted as macroscopic colonies by examination on an inverted microscope at ×40. For the sequential observations of individual colonies, location of newly emerging colonies within the dishes was recorded twice a week, and then presence of the colonies was inspected.

Detection of Transcript of c-kit Ligand Gene after RT-PCR Amplification. Total RNA was prepared from 10^7 cells of parental PA6 clone, each of its subclones, or ST2 cell line by the guanidinium/CsCl method. The sequence of the synthetic oligonucleotide was: rat c-*kit* ligand, 5′ primer; 5′-ATGAAGAAGACACAAACTTGGATT-3′, 3′ primer; 5′-AATATTTGAAAACTTGTCCAGAAG-3′, mouse β-actin, 5′ primer; 5′-TCGTGCGTGACATCAAAGAG-3′, 3′ primer; 5′-TGGACAGTGAGGCCAGGATG-3′. These primers were synthesized with a PCR-Mate DNA synthesizer (391; Applied Biosystems, Inc., Foster City, CA). The isolated total RNA was reverse transcribed in a total volume of 20 μl in a buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 10 mM dNTP mixture, 100 pmol random hexamer oligonucleotides (Takara Shuzo, Kyoto, Japan), and 200 U Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD). dsDNA was then synthesized from the ssDNA with 1 U of *Thermus aquaticus* (Taq) polymerase (Takara Shuzo), and the two pairs of oligonucleotide primers by repeating 25–30 cycles of the PCR on a PCR Thermocycler (1000; Perkin Elmer Cetus, Norwalk, CT). Each cycle included denaturation at 94°C for 1 min, reannealing of the primer and fragmentation at 55°C for 2 min, and polymerization of 75°C for 2 min. A fraction of each sample of the amplified DNA was subjected to 5% PAGE. DNA bands were stained with ethidium bromide.

Results

Ability of PA6 Cells to Support the Proliferation of Hemopoietic Stem Cells Capable of Reconstituting In Vivo Erythropoiesis. Although the number of day 12 CFU-S increases ~12-fold after 7 d of coculture with PA6 cells (28), and hemopoiesis within the cocultures continued for at least 15 wk (data not shown), it had not been assessed whether the preadipocytes can support the proliferation of the hemopoietic stem cells capable of reconstituting in vivo hemopoiesis.

To examine the growth of such stem cells within the cocultures, we first enumerated by limiting dilution analysis the frequency of the stem cells capable of sustaining hemopoiesis for up to 6 wk. Varying numbers of bone marrow cells were cocultured with PA6 cells. On days 28, 35, and 42 of coculture, nonadherent cells were harvested and counted. Dishes containing >5 × 10^5 nonadherent hemopoietic cells were judged as hemopoiesis positive. Most of the negative dishes contained much fewer nonadherent cells than the number needed to meet our criterion, and their frequency increased as the culture period was prolonged, reflecting the decrease

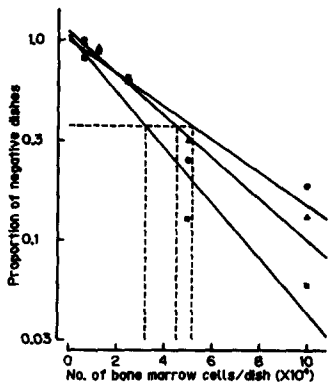

Figure 1. Limiting dilution analysis of the frequency of hemopoietic stem cells capable of sustaining hemopoiesis within the cocultures with PA6 cells. Varying numbers of bone marrow cells were inoculated onto PA6 cell layers established in 35-mm dishes and cocultured for 28 (■), 35 (▲), and 42 (●) d. Dishes containing 5 × 10^5 nonadherent cells or more were judged as hemopoiesis positive. Each cell dilution consisted of 15–20 dishes.

in the number of the stem cells sustaining their proliferative capacity (Fig. 1).

The proportion of the negative dishes was plotted against the inoculated number of bone marrow cells. From the data obtained on day 28, 35, and 42 of coculture, straight regression lines were obtained in a semilogarithmic plot that intercepted the ordinate at 1.04, 1.08, and 0.99, respectively (Fig. 1). The frequency of the stem cells that sustained hemopoiesis within the cocultures for 28, 35, and 42 d was estimated to be 1/3.2 × 10^4, 1/4.5 × 10^4, and 1/5.3 × 10^4 bone marrow cells, respectively.

In the group of dishes into which bone marrow cells had been inoculated at 2.5 × 10^4 cells/dish, 7 of 19 dishes (37%) were positive for hemopoiesis on day 42 of coculture. We harvested hemopoietic cells associated with adherent cell layers of these hemopoiesis-positive dishes individually and injected them into each of seven WBB6F$_1$-*W/W^v* mice. Fig. 2 shows electrophoretic patterns of hemoglobin from these recipient mice at 24 wk after the injection. Hemoglobin from two of these mice (lanes *4* and *5*) showed a pure Hbb^s/Hbb^s pattern, indicating that whole erythropoiesis of these mice had been reconstituted by the hemopoietic stem cells derived from donor C57BL/6 mice. In these mice, the pure Hbb^s/Hbb^s pattern had been observed already at 8 wk after the injection. In one mouse (lane *9*), erythropoiesis was partially recon-

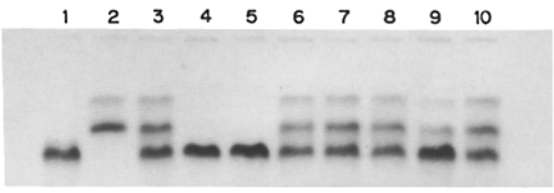

Figure 2. Electrophoretic patterns of hemoglobin from WBB6F$_1$-*W/W^v* mice injected with hemopoietic cells harvested from the cocultures of bone marrow cells and PA6 cells. In the experiment shown in Fig. 1, in the group of day 42 cocultures in which bone marrow cells had been inoculated at 2.5 × 10^4 cells/dish, 7 of 19 dishes were hemopoiesis positive. Hemopoietic cells were harvested from each hemopoiesis-positive dish and injected into each of seven WBB6F$_1$-*W/W^v* mice. Peripheral blood was obtained from a C57BL/6 mouse (lane *1*), a WB/Re mouse (lane *2*), an uninjected WBB6F$_1$-*W/W^v* mouse (lane *3*), or seven recipient WBB6F$_1$-*W/W^v* mice (lanes *4–10*) at 24 wk after the injection.

stituted by the injected stem cell(s). Presence of donor type hemoglobin, as well as that of the recipient type, had been clearly noticed from 16 wk after the injection. These results demonstrate that PA6 cells can support the proliferation of hemopoietic stem cells having extensive proliferative capacity.

Effect of an Anti-c-kit mAb on the Hemopoiesis Supported by PA6 Cells. The mAb ACK2, recognizing an extracellular domain of the c-*kit* receptor molecule, has been strongly suggested to act as an antagonist and to be cytostatic rather than cytotoxic for the cells expressing the antigen (22, 23). To elucidate the functional role of the c-*kit* molecule in the proliferation of hemopoietic stem cells in our coculture system, we first examined the effect of the antibody on the production of nonadherent hemopoietic cells. Purified ACK2 was added to the cocultures at varying concentrations at the onset of the cocultures, and then the cocultures were continued with antibody-free medium from day 4.

As shown in Fig. 3, ACK2 dose-dependently reduced the production of nonadherent hemopoietic cells. In the cocultures treated with 10 μg/ml ACK2, nonadherent cell production rapidly declined to the level as low as 1–2% of that in the untreated cocultures during the second week of coculture. During the fourth and fifth weeks, however, hemopoiesis within the ACK2-treated cocultures dramatically became active, and production of nonadherent cells reached the level of that in the untreated cocultures. The number of day 12 CFU-S decreased to ~6% of the inoculated number after 4 d of coculture in the presence of 10 μg/ml ACK2, whereas the CFU-S number in the untreated cocultures increased about threefold over the same period (Fig. 4).

To exclude the possibility that the recovery of hemopoiesis was due to insufficiency of the ACK2 treatment, we repeatedly added ACK2 to the cocultures on days 0, 4, 7, and 11 of coculture, and then the cocultures were continued with antibody-free medium from day 14. Even this prolonged ACK2 treatment could not abrogate the recovery of hemopoiesis, although the reduction of hemopoiesis was more severe and recovery delayed (Fig. 5). Next, we examined the effect of pretreatment of bone marrow cells with the antibody. Bone marrow cells were incubated with ACK2 at 37°C for 1 h before the ACK2 treatment of the cocultures for the initial 4 d. The stem cells responsible for the recovery of hemopoiesis still could survive this intense ACK2 treatment (Table 1). These results strongly suggest that the c-*kit* molecule is not functionally requisite for the survival and proliferation of a certain population of hemopoietic stem cells.

Colony Formation of Hemopoietic Stem Cells within the Cocultures of Bone Marrow Cells and PA6 Cells and Sensitivity of the Clonogenic Cells to ACK2 Treatment. To elucidate the proliferative properties of the hemopoietic stem cells surviving the

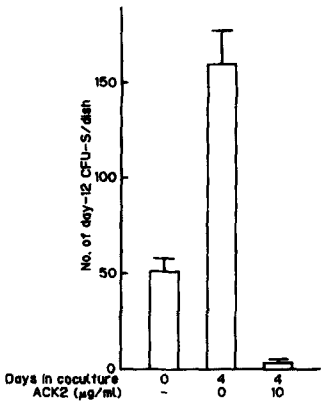

Figure 4. Effect of ACK2 on the number of CFU-S within the cocultures of bone marrow cells and PA6 cells. Bone marrow cells were inoculated at 3 × 10^5 cells/35-mm dish onto PA6 cell layers and cocultured in the absence or presence of 10 μg/ml ACK2 antibody for 4 d. Hemopoietic cells harvested from the cocultures or freshly isolated from bone marrow were injected into lethally irradiated mice. At 12 d after the injection, spleen colonies were counted. Each column represents the mean ± SD of counts from eight spleens.

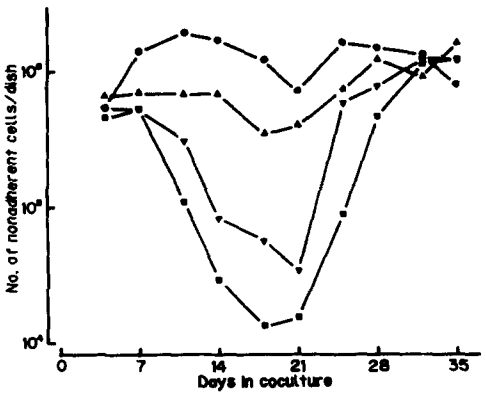

Figure 3. Effect of anti-c-*kit* mAb on the hemopoiesis supported by PA6 cells. Bone marrow cells were inoculated at 3 × 10^5 cells/35-mm dish onto PA6 cell layers and cocultured for 35 d. Purified ACK2 mAb was added to the cocultures at the concentration of 0 (●), 0.1 (▲), 1 (▼), or 10 (■) μg/ml at the onset of the cocultures. On day 4, the cocultures were washed three times with medium. Then the cocultures were continued with antibody-free medium by changing of the medium twice a week. At every medium change, nonadherent cells were harvested from at least four dishes by gentle pipetting and rinsing once with medium, pooled, and counted.

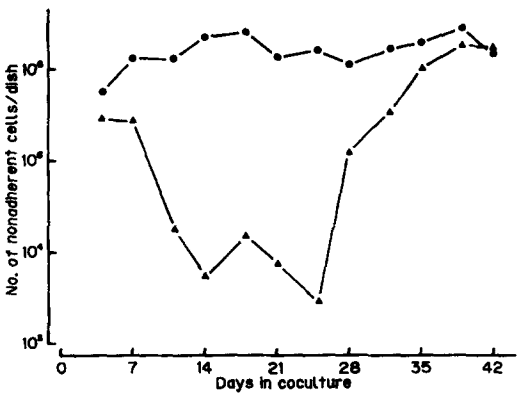

Figure 5. Effect of prolonged ACK2 treatment on the hemopoiesis supported by PA6 cells. Bone marrow cells were inoculated at 3 × 10^5 cells/35-mm dish onto PA6 cell layers and cocultured in the absence (●) or presence (▲) of 10 μg/ml ACK2 for 14 d with medium change twice a week. Then, after having been washed with medium, the cocultures were continued with antibody-free medium. At every medium change, nonadherent cells were counted as described in the legend to Fig. 3.

Table 1. *Effect of Pretreatment of Bone Marrow Cells with ACK2 on the Survival of Hemopoietic Stem Cells*

ACK2 treatment		No. of nonadherent cells/dish*	
Preincubation‡	Coculture§	On day 14	On day 28
–	–	3.5×10^6	2.7×10^6
–	+	5.0×10^4	2.1×10^6
+	+	5.0×10^4	1.2×10^6

* Nonadherent cells were harvested and counted as described in the legend to Fig. 3.

‡ Bone marrow cells were suspended at 2×10^5 cell/ml in the medium and incubated at 37°C for 1 h in the absence or presence of 10 μg/ml ACK2.

§ Bone marrow cells were inoculated at 3×10^5 cells/35-mm dish onto PA6 cell layers and cocultured in the absence or presence of 10 μg/ml ACK2 for the initial 4 d. Then, after having been washed with medium, the cocultures were continued with antibody-free medium, which was changed twice a week.

ACK2 treatment and to compare them with those of ACK2 sensitive stem cells, we developed a colony assay system of hemopoietic stem cells by modifying our coculture system. Since cell-to-cell contact with PA6 cells is a requisite for the proliferation of CFU-S (27), and most of the CFU-S and progenitor cells of neutrophils and macrophages are distributed within the PA6 cell layers (28), we first cocultured bone marrow cells and PA6 cells with liquid medium for 2–4 d, and then the adherent cell layers were covered with a collagen gel. These semisolidified cocultures could be continued for up to 6–7 wk by changing of the medium that was added onto the gel.

Colonies having a diameter of 2 mm or more and containing immature hemopoietic cells and/or both neutrophils and macrophages were counted as macroscopic colonies (Fig. 6). At their early stage of development, the colonies contained only immature hemopoietic cells, and later these cells differentiated into neutrophils or macrophages. Some of the neutrophils moved up to the liquid phase. Colonies tended to become larger with increasing time in coculture, and their diameter reached up to 6 mm (Fig. 6, *right*). When the colonies came to the end of their existence, the neutrophils in the colonies died simultaneously. We judged such colonies as having disappeared, although macrophages in these colonies survived for a long time after the loss of neutrophils and gradually dispersed. Length of the period of liquid culture within the range of 2 and 4 d did not affect either number or size of the colonies (data not shown).

Fig. 7 shows the time course of change in the number of macroscopic colonies in the cocultures in which bone marrow cells were inoculated at 2×10^5 cells/60-mm dish. In the untreated cocultures, many macroscopic colonies were already detected on day 7. At this inoculum size, however, counting of the colonies was hampered by the presence of many small colonies. By day 11, however, most of the small colonies had disappeared, and ~70 discrete macroscopic colonies were detected per dish. The colony number decreased to about half at every twice-a-week counting until day 32. Then as few as 1.5–2 colonies were observed per dish until day 42.

When the cocultures were treated with 10 μg/ml ACK2 during the 4-d period of liquid culture, 1.6 ± 1.3 macroscopic colonies first developed per dish on day 11. Then the colony number never exceeded six colonies/dish throughout our observation period up to day 42 (Fig. 7). Nevertheless, at later than day 28, the colony number in the ACK2-treated cocultures was comparable to that in the untreated cocultures. Colonies developed within the ACK2-treated cocultures tended to become larger than those in the untreated cocultures, and the diameter of some colonies reached up to 10 mm at later than day 25. These results demonstrate that hemopoietic stem cells forming colonies earlier within the cocultures are selectively killed by the ACK2 treatment.

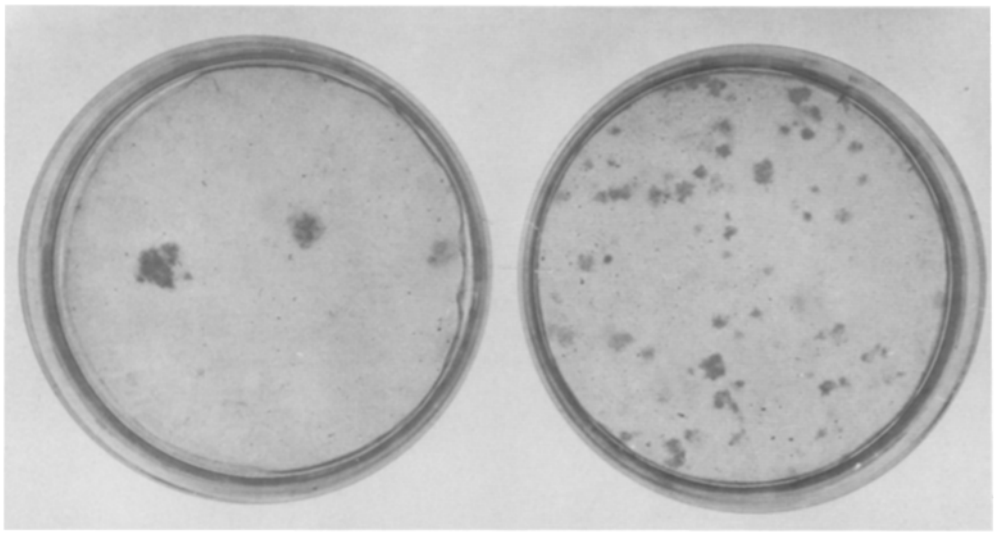

Figure 6. Macroscopic colonies formed in the semisolidified cocultures. Bone marrow cells were inoculated at 2×10^5 cells/60-mm dish onto PA6 cell layers and cocultured in liquid medium. On day 2, adherent cell layers were covered with a collagen gel. Then the cocultures were continued by changing of the medium added onto the gel twice a week for 14 (*left*) or 28 (*right*) d. Then the cocultures were dehydrated by covering of the gel with a lens paper and filter papers, fixed with 10% formalin, and stained with hematoxylin.

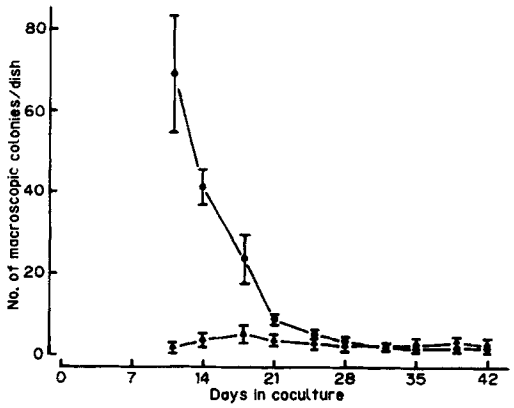

Figure 7. Time course of the change in the number of macroscopic colonies in the untreated and ACK2-treated bone marrow/PA6 cocultures. Bone marrow cells were inoculated at 2 × 10^5 cells/60-mm dish onto PA6 cell layers and cocultured in the absence (●) or presence (▲) of 10 μg/ml ACK2 with liquid medium for 4 d. Then, after having been washed with medium, the adherent cell layers were covered with a collagen gel. The cocultures were continued by changing of the medium, added onto the gel, twice a week. Colonies having a diameter of 2 mm or more and containing immature hemopoietic cells and/or both neutrophils and macrophages were counted as macroscopic colonies. Each point represents the mean ± SD of counts from at least four dishes.

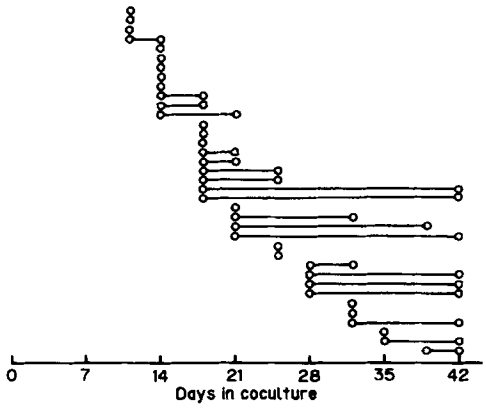

Figure 8. Development of individual macroscopic colonies and their persistence in the ACK2-treated cocultures. Conditions of cocultures of bone marrow cells and PA6 cells and ACK2 treatment were similar to those described in the legend to Fig. 7. Twice a week, location of newly emerging colonies within three dishes was recorded, and their persistence was inspected.

Table 2. *Development of Macroscopic Colonies and Their Disappearance within the Cocultures of Bone Marrow Cells and PA6 Cells*

Period of persistence of macroscopic colonies	No. of colonies*
Day 7	72
Day 7–11	12
Day 7–14	4
Day 7–18	1
Day 11	18
Day 11–14	11
Day 11–18	3
Day 11–25	1
Day 14	10
Day 14–18	2
Day 14–25	1
Day 18	4
Day 21–25	1
Day 32–42	1

Bone marrow cells were inoculated onto PA6 cell layers at 5 × 10^4 cells/60-mm dish and cocultured in liquid medium for 2 d. Then adherent cell layers were covered with a collagen gel, and cocultures were continued by changing of the medium, added onto the gel up to day 42.
* Twice a week, location of newly emerging macroscopic colonies within two dishes was recorded, and their persistence was inspected.

Next, to disclose the proliferative properties of the stem cells forming colonies in our coculture system more precisely, we sequentially observed the development of individual macroscopic colonies and their disappearance by recording the location of newly emerging colonies twice a week and inspecting their persistence. To facilitate the identification of the colonies formed in the untreated cocultures, we reduced inoculum size of the bone marrow cells to 5 × 10^4 cells/dish. Adherent cell layers were covered with a collagen gel on day 2 of coculture. As shown in Table 2, a total of 141 macroscopic colonies were formed in two such dishes during a 6-wk observation period. 89 colonies (63%) reached macroscopic size on day 7. 104 colonies (74%) were detected at only one observation time. Only 11 colonies (8%) were detected at more than three observation times, i.e., for >8

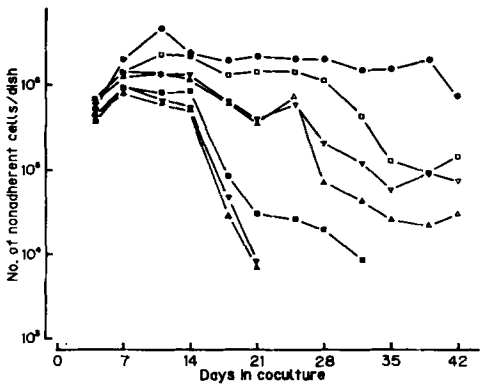

Figure 9. Time course of the hemopoiesis supported by the cells of subclones of PA6 clone. Cell layers of PA6 clone (●), subclone 2 (▲), subclone 12 (▼), subclone 14 (■), a mixture of PA6 clone and subclone 2 (△), a mixture of PA6 clone and subclone 12 (▽)), or a mixture of PA6 clone and subclone 14 (□) were established in 35-mm dishes. Bone marrow cells were inoculated onto these cell layers and cocultured, and nonadherent cells were counted as described in the legend to Fig. 3.

d; and only one colony (0.7%), arising on day 32, persisted until day 42. These results demonstrate that, in the untreated cocultures, most of the macroscopic colonies are formed by hemopoietic stem cells having limited proliferative capacity.

Fig. 8 illustrates the appearance of individual macroscopic colonies and their persistence in the cocultures treated with 10 μg/ml ACK2 for the initial 4 d. Bone marrow cells were inoculated at 2 × 10^5 cells/dish. A total of 37 macroscopic colonies were formed in three such dishes during 6 wk of observation, showing that as many as ~95% of the cells capable of forming macroscopic colonies in the present colony assay system were killed by the ACK2 treatment. New macroscopic colonies emerged until as late as 39 d after inoculation. 13 colonies (35%) were observed at more than three observation times, while 17 colonies (46%) were detected on one occasion only. Nine colonies (24%) persisted until end of the observation period. These colonies reached a macroscopic size on or later than day 18. These observations clearly demonstrate that hemopoietic stem cells having higher proliferative capacity preferentially survive the ACK2 treatment.

Properties of Subclones of PA6 Cell Line That Are Incapable of Supporting Long-Term Hemopoiesis. Above observations of the fate of individual colonies indicate that the apparently constant level of hemopoietic cell production in our coculture system is maintained by successive bursts of the proliferation and differentiation of hemopoietic stem cells having varying proliferative properties. Hemopoietic cell production by the stem cells having higher proliferative capacity tended to occur later. To further explore the mechanism through which PA6 cells support the proliferation of the hemopoietic stem cells having extensive proliferative capacity, we isolated subclones incapable of supporting long-term hemopoiesis from PA6 clone.

Table 3. *Inability of rmc-kit Ligand to Cure the Defect of Subclones of PA6 Clone*

Cell layers	rmc-*kit* ligand	No. of nonadherent cells/dish* On day 14	On day 21
PA6 clone	–	2.0 × 10^6	1.5 × 10^6
	+	2.5 × 10^6	1.8 × 10^6
Subclone 2	–	4.2 × 10^5	3.2 × 10^4
	+	6.0 × 10^5	1.7 × 10^4
Subclone 12	–	6.6 × 10^5	1.7 × 10^4
	+	8.7 × 10^5	4.3 × 10^4
Subclone 14	–	9.0 × 10^5	7.0 × 10^5
	+	1.6 × 10^6	1.4 × 10^5

Bone marrow cells were inoculated at 3 × 10^5 cells/35-mm dish onto the cell layers of PA6 clone or one of its subclones and cocultured in the absence or presence of 50 ng/ml partially purified rmc-*kit* ligand.
* Nonadherent cells were harvested and counted as described in the legend to Fig. 3.

When bone marrow cells were cocultured with the cells of subclone 2, 12, or 14, active nonadherent cell production was sustained for only 2 wk, and then hemopoiesis declined rapidly, while the low level of hemopoiesis continued for a significantly prolonged period in the cocultures with subclone 14 cells (Fig. 9, Table 3). These observations demonstrate that these subclones have lost the capacity to support the proliferation of the hemopoietic stem cells having extensive proliferative capacity. All of these subclones retained the capacity to differentiate into adipocytes (26) at low frequency and to support megakaryopoiesis (27) as actively as parental PA6 clone (data not shown). Subclones 2 and 12 also retained the capacity to support osteoclast differentiation (34) as actively as PA6 clone, but subclone 14 had completely lost such capacity (data not shown). These observations suggest that these subclones of PA6 clone have lost the expression of a small number of genes.

Then, we mixed equal numbers of parental PA6 cells and each of the subclone cells and established cell layers. When bone marrow cells were inoculated onto these mixed cell layers and cocultured, the level of nonadherent cell production was intermediate between that in the cocultures with PA6 cells and that with subclone cells (Fig. 9). Hemopoiesis within the cocultures with the mixture of PA6 cells and subclone cells declined slowly after the initial 2 wk of active hemopoiesis but continued for a significantly longer period than that supported by any of subclone cells. Therefore, inability of the subclone cells to support long-term hemopoiesis is unlikely to be due to their production of inhibitory molecule(s). Although the reason why the mixed cells were not as active as PA6 cells alone in the support of hemopoiesis is not clear, hemopoietic stem cells may have difficulty in maintaining their proliferative capacity, if the stem cells do not have the ability to selectively contact parental PA6 clone cells.

To determine whether the inability of the subclone cells to support the proliferation of primitive hemopoietic stem cells is due to their lack of gene expression of the c-*kit* ligand, we prepared mRNA from PA6 cells, each of subclone cells, and ST2 cells and amplified transcripts of c-*kit* ligand gene and β-actin gene by the RT-PCR method. As shown in Fig. 10, no significant difference in the level of the expression of

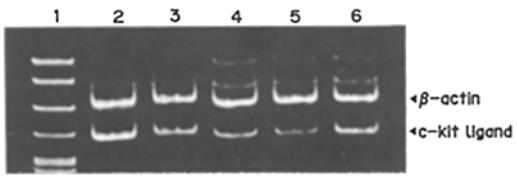

Figure 10. Electrophoretic analysis of the products of RT-PCR amplification using oligonucleotide primers of c-*kit* ligand and β-actin. Total RNA isolated from the indicated cells was used as template for cDNA synthesis and subsequent RT-PCR amplification in combination with two pairs of oligonucleotide primers: rat c-*kit* ligand and mouse β-actin. The products were separated by electrophoresis, and bands of c-*kit* ligand (272 bp) and β-actin (430 bp) were visualized by ethidium bromide staining. Lane *1*, molecular weight markers; lane *2*, ST2 cells; lane *3*, PA6 cells; lane *4*, subclone 2 cells; lane *5*, subclone 12 cells; lane *6*, subclone 14 cells.

mRNA for the c-*kit* ligand was noticeable among the cells of clone PA6 or of any of its subclones. ST2 cells, capable of supporting the proliferation of hemopoietic stem cells and their differentiation into both myeloid and B lymphoid cells (29, 30), also expressed c-*kit* ligand mRNA. Furthermore, we exogenously supplied 50 ng/ml partially purified rmc-*kit* ligand to the cocultures of bone marrow cells and the cells of PA6 clone or one of the subclones. As shown in Table 3, the rmc-*kit* ligand slightly enhanced the production of nonadherent cells during first 2 wk of coculture but did not cure the defect of the subclone cells in the ability to support long-term hemopoiesis. These results seem to exclude the possibility that the ability of PA6 cells to support the proliferation of the hemopoietic stem cells having extensive proliferative capacity is not solely borne by their expression of c-*kit* ligand.

Discussion

In the present study, our first question was whether hemopoietic stem cells having extensive proliferative capacity can proliferate within cocultures of bone marrow cells and PA6 cells. We first cocultured varying numbers of bone marrow cells with the preadipocytes. Frequency of the stem cells capable of sustaining hemopoiesis within the cocultures for 6 wk was estimated to be $1/5.3 \times 10^4$ bone marrow cells by limiting dilution analysis. We next examined the presence of the primitive hemopoietic stem cells within the hemopoiesis-positive dishes into which bone marrow cells had been inoculated at 2.5×10^4 cells/dish. Hemopoietic cells harvested from each of seven such dishes were injected into each of seven WBB6F$_1$-*W/W^v* mice. At 24 wk after the injection, erythropoiesis was reconstituted completely in two recipient mice and partially in one mouse by the injected stem cells. These results demonstrate that PA6 cells can support the proliferation of the hemopoietic stem cells having extensive proliferative capacity. It is unlikely that dormant primitive stem cells may have accidentally strayed into the hemopoiesis-positive dishes, since frequency of more primitive hemopoietic stem cells must be lower than that of more differentiated ones.

Although, judging from the statistical point of view, the sample number in our present experiment is not sufficient, the frequency of the stem cells capable of sustaining hemopoiesis throughout our present experiment, i.e., 6 wk in vitro and 24 wk in vivo, can be roughly estimated. Since, in the group of dishes into which bone marrow cells had been inoculated at 2.5×10^4 cells/dish, hemopoietic stem cells having long-term reconstituting capacity were detected in 3 of 19 dishes (16%), the mean number of such stem cells/dish is estimated to be 0.17 (35). Therefore, the concentration of the primitive hemopoietic stem cells in the starting bone marrow cell population is calculated to be $6.8/10^6$ cells. Nakano et al. (7) injected varying numbers of bone marrow cells freshly isolated from C57BL/6 mice into WBB6F$_1$-*W/W^v* mice and estimated by limiting dilution analysis the concentration of the primitive hemopoietic stem cells to be $9/10^6$ bone marrow cells. By comparison of these estimations, ~75% of the primitive hemopoietic stem cells are supposed to have sustained their proliferative capacity during 6 wk of the coculture with PA6 cells, showing that PA6 cells can provide a fairly suitable microenvironment for the proliferation of primitive hemopoietic stem cells.

To explore the proliferation of individual hemopoietic stem cells in our coculture system, we developed a colony assay system of the stem cells. Bone marrow cells and PA6 cells were initially cocultured in liquid medium for 2–4 d to allow the stem cells to enter into the PA6 cell layers (27, 28). Then the adherent cell layers were covered with a collagen gel to restrict the movement of hemopoietic cells. Clonality of the colonies may be obscured during the initial period of liquid culture. However, hemopoietic progenitor cells capable of forming multilineage colonies in methylcellulose cultures are strongly suggested to be in G_0 state for a variable period after inoculation, and, when triggered into cell cycle, they proliferate at relatively constant doubling rates (36). Nakano et al. (7) reported that hemopoietic stem cells capable of reconstituting in vivo hemopoiesis are not in S phase. It appears to take only 4–5 d for the colonies in our colony assay system to reach macroscopic size, since population doubling time of the cells within the colonies was 7–8 h (H. Kodama, unpublished observations). Accordingly, many of the cells forming macroscopic colonies are considered to have stayed in G_0 state during the liquid culture period. Even if some of the colony-forming cells entered into the cell cycle during the liquid culture period, their movement would be restricted, since they proliferate exclusively within the adherent cell layers in close contact with PA6 cells (27, 28).

The time course of colony formation in our coculture system seems to be equivalent to that of spleen colony formation, since the population doubling time of the cells within the colonies was similar to the shortest cell-cycle time of mammalian cells (37). Therefore, macroscopic colonies detected at around the second week of coculture probably correspond to the spleen colonies. The number of the in vitro colonies decreased to about half at every twice-a-week counting from day 11 to 32. Consequently, colonies detected on day 42 were as few as ~2.5% of those detected on day 11, indicating that most of the colonies are formed by the hemopoietic stem cells having limited proliferative capacity.

The frequency of the hemopoietic stem cells estimated by the number of macroscopic colonies on day 42 of coculture was about half of that estimated by limiting dilution analysis. In the colony assay system, hemopoietic cells were restricted to grow within a small area, while the cells could disperse over the entire surface of 35-mm dishes in the limiting dilution assay. Competition among hemopoietic cells for contact with PA6 cells may occur within the colonies. If this is the case, it may be difficult for stem cells to maintain their proliferative capacity in the colony assay system. Despite this drawback, our present colony assay system enabled us to dissect the heterogeneous population of hemopoietic stem cells having varying proliferative capacities.

We found by sequential observations of individual macroscopic colonies that new macroscopic colonies continuously

emerged until as late as 39 d after inoculation. Such an asynchrony in the development of colonies has been already observed in the colony formation in both spleens (38, 39) and methylcellulose cultures (36). After varying periods of sustained hemopoiesis, most of the colonies suddenly disappeared, similarly as observed in the spleen colonies (38, 39). Consequently, only 8% of the macroscopic colonies were detected at more than three twice-a-week observation times, i.e., for >8 d; and only one out of a total of 141 colonies persisted until the end of the 42 d of our observation period. Although the colonies emerging later tended to persist for a longer period, the fate of individual colonies was unpredictable. Both the asynchrony in the development of colonies and the unpredictability of their fate seem to indicate the stochastic nature of the responses of the hemopoietic stem cells to environmental stimuli (36, 40, 41). Nevertheless, no colonies having reached macroscopic size before day 18 persisted until day 42, suggesting that hemopoietic stem cells capable of reconstituting in vivo hemopoiesis may not form macroscopic colonies in spleens at least within 14 d after injection.

Our second question was whether PA6 cells support the proliferation of primitive hemopoietic stem cells through a mechanism involving the c-*kit* receptor and its ligand. For this purpose, we first utilized a mAb, ACK2, recognizing an extracellular domain of the c-*kit* molecule, since this antibody has been strongly suggested to antagonistically block the function of the c-*kit* receptor (22, 23). Addition of the antibody to the cocultures of bone marrow cells and PA6 cells resulted in the severe reduction in the hemopoietic cell production. Only ~6% of day 12 CFU-S survived after 4 d of coculture in the presence of ACK2, while the CFU-S number in the untreated cocultures increased about threefold during the same period. These results are consistent with the findings that injection of ACK2 into mice results in a simultaneous disappearance of CFU-S and hemopoietic progenitor cells assayable in vitro by using various hemopoietic factors (23), and that colony formation of CFU-S is inhibited by the injection of the antibody into recipient mice (24).

However, when the cocultures were continued with antibody-free medium after 4 or 14 d of the ACK2 treatment, hemopoiesis dramatically recovered to the level of that in the untreated cocultures. Our colony assay system enabled us to clarify the difference of the hemopoietic stem cells surviving the ACK2 treatment in their proliferative properties from those of the majority of the stem cells. Only ~5% of the cells forming macroscopic colonies in the cocultures of bone marrow cells and PA6 cells survived the 4 d of ACK2 treatment. On day 11 of coculture, the number of macroscopic colonies detected in the ACK2-treated cocultures was ~2.5% of that in the untreated cocultures. However, at later than day 28, colony number in the ACK2-treated cocultures was comparable to that in the untreated cocultures. The frequency of the macroscopic colonies persisting for >8 d in the ACK2-treated cocultures was fourfold higher than that in the untreated cocultures. Furthermore, as many as 24% of the colonies persisted until day 42 in the ACK2-treated cocultures, while only 1 of 141 colonies persisted in the untreated cocultures. These results clearly demonstrate that hemopoietic stem cells having higher proliferative capacity preferentially survive the ACK2 treatment.

The above finding that primitive hemopoietic stem cells are resistant to ACK2 treatment does not necessarily mean that the c-*kit* molecule is not expressed on such stem cells, since the ACK2 antibody has been strongly suggested to be cytostatic rather than cytotoxic for the cells expressing its antigen (22, 23). In fact, Okada et al. (24) and Ikuta and Weissman (25) found that only the cells of the lineage marker–negative and c-*kit*-positive fraction of bone marrow cells can reconstitute the lymphohemopoietic system of lethally irradiated mice. Although CFU-S exist exclusively in the c-*kit*-positive fraction (24, 25), a small but significant fraction of day 13 CFU-S is resistant to ACK2 (23), and colony formation of day 12 CFU-S is less effectively inhibited by the injection of ACK2 into recipient mice than that of day 8 CFU-S (24). Therefore, it is likely that the c-*kit* molecule is expressed on all the hemopoietic stem cells but that the stem cells at the earlier stage are less dependent on the signal transduction through the c-*kit* receptor. Recently, Ikuta and Weissman (25) reported that *Sl/Sl* homozygote fetuses, which lack genes to encode functional c-*kit* ligand (20, 21), have 30–40% of the number of hemopoietic stem cells in their liver when compared with normal littermates, and that the absolute number of hemopoietic stem cells increases during fetal development in the *Sl/Sl* mice.

Next, we isolated three subclones incapable of supporting long-term hemopoiesis from the PA6 clone. When bone marrow cells were cocultured with the cells of these subclones, active hemopoiesis continued for only 2 wk, indicating that the subclone cells have lost the capacity to support the proliferation of primitive hemopoietic stem cells. These three defective subclones were found by testing only 17 subclones isolated from highly passaged PA6 clone. Mixed cell layers comprising the PA6 clone and any of the defective subclones supported hemopoiesis for a significantly longer period than 2 wk. Therefore, inability of the subclone cells to support long-term hemopoiesis is unlikely to have resulted from either mutation in some gene or their production of some inhibitory molecule(s).

Production of functional c-*kit* ligand by the subclone cells seems to be apparent, since active hemopoiesis in the cocultures of bone marrow cells and the subclone cells continued significantly longer than that in the ACK2-treated cocultures of bone marrow cells and PA6 cells. In fact, we failed to detect any significant difference in the level of expression of mRNA for c-*kit* ligand between the cells of PA6 clone and any of its subclones. The defect of the subclone cells was not cured by the exogenous supply of rmc-*kit* ligand. These results suggest that merely expressing c-*kit* ligand is not enough for PA6 cells to support the proliferation of primitive hemopoietic stem cells.

In conclusion, our present study has demonstrated that a combination of our in vitro colony assay system and ACK2

mAb provides a powerful tool for the analysis of the proliferative properties of primitive hemopoietic stem cells. The results obtained by use of both the antagonistic ACK2 mAb and the defective subclones of PA6 clone consistently suggest that the mechanism involving c-*kit* receptor and its ligand does not play a major role in the survival and proliferation of primitive hemopoietic stem cells.

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