

## Expression and Function of *c-kit* in Hemopoietic Progenitor Cells

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### Summary

The expression and function of a receptor tyrosine kinase, *c-kit*, in the adult bone marrow of the mouse were investigated by using monoclonal antibodies (mAbs) against the extracellular domain of murine *c-kit*. In adult C57BL/6 mouse, 7.8% of total bone marrow cells express *c-kit* on their surface. Half of the *c-kit*<sup>+</sup> cells do not express lineage markers including Mac-1, Gr-1, TER-119, and B220, while the remainder coexpress myeloid lineage markers such as Mac-1 and Gr-1. After *c-kit*<sup>+</sup> cells were removed from the bone marrow cell preparation, hemopoietic progenitor cells reactive to IL-3, GM-CSF, or M-CSF and also those which give rise to spleen colonies in irradiated recipients disappeared almost completely. Thus, most hemopoietic progenitors in the adult bone marrow express *c-kit*. To investigate whether or not *c-kit* has any role in the hemopoiesis of adult bone marrow, we took the advantage of one of the anti-*c-kit* mAbs that can antagonize the function of *c-kit*. As early as two days after the injection of 1 milligram of an antagonistic antibody, ACK2, almost all hemopoietic progenitor cells disappeared from the bone marrow, which eventually resulted in the absence of mature myeloid and erythroid cells in the bone marrow. These results provide direct evidence that *c-kit* is an essential molecule for constitutive intramarrow hemopoiesis, especially for the self-renewal of hemopoietic progenitor cells at various stages of differentiation.

The protooncogene *c-kit* encodes a receptor tyrosine kinase belonging to the platelet derived growth factor (PDGF)<sup>1</sup>/CSF-1 receptor subfamily (1). Recent studies have shown that *c-kit* is allelic with the dominant spotting locus (*W*) of mice (2, 3). Since mice homozygous with the *W* allele which encodes a nonfunctional *c-kit* gene product die perinatally of severe anemia (4), it is clear that *c-kit* and its ligand play an essential role in intramarrow hemopoiesis in the adult mouse. More recently, a gene encoding the ligand for *c-kit* was cloned by several groups (5–9). This ligand was demonstrated to have colony stimulating activity of multipotent progenitors (9–11). Thus far, this newest hemopoietic growth factor and CSF-1 are the only two factors whose actual roles in vivo are well understood, owing to the availability of the mice which are defective in these genes (12–14).

Despite the considerable understanding of the essential role of *c-kit* and its ligand on the regulation of constitutive hemopoiesis in adult bone marrow (15, 16), several questions remain to be resolved. First, it is not clear which types of intramarrow hemopoietic cells express *c-kit*, although expression of *c-kit* on the surface of mast cells has been clearly shown (17). To closely examine the expression of *c-kit* in bone marrow cells, it is necessary to produce mAbs against the extracellular domain of *c-kit*. Secondly, recent studies have suggested that multipotent stem cells, erythroid progenitors and some B lineage progenitors can be stimulated to form colonies in vitro by the recombinant *c-kit* ligand (5, 9–11). However, this does not necessarily mean that the maintenance of these progenitor cells in the bone marrow is actually dependent on *c-kit*. If a mAb which can antagonize the function of *c-kit* is obtained, an in vivo blocking experiment may resolve this question. This kind of experiment is important for such a molecule as *c-kit* whose expression does not necessarily indicate that it is functioning. For example, although *c-kit* has

<sup>1</sup> Abbreviations used in this paper: APC, allophycocyanin; CFC, colony-forming cell; GM-CSF, granulocyte macrophage colony-stimulating factor; PDGF, platelet-derived growth factor.

been shown to be expressed in the brain (3, 18), development of the central nervous system appears to be normal even in the *W/W* mouse, which does not express functional *c-kit* at all.

Recently, we developed mAbs against the extracellular domain of *c-kit*, some of which can block the function of *c-kit* both in vitro and in vivo (46). An antagonistic anti-*c-kit* mAb injected into pregnant mice could be transported to the embryo through the placenta, and was able to block the colonization of melanocyte precursors into the epidermal layer of the skin, thereby producing unpigmented offspring from a pigmented mouse. In the present study, we used these anti-*c-kit* mAbs and attempted to determine the expression and function of *c-kit* in hemopoietic cell progenitors. Our results demonstrate that *c-kit* is expressed on almost all hemopoietic cell progenitors clonable in vitro by various soluble hemopoietic growth factors and on most spleen colony-forming cells (CFCs). We also show that this receptor molecule actually functions in these cells in vivo.

## Materials and Methods

**Mice.** C57BL/6 mice and *WB-W/+* mice were purchased from Japan SLC Inc. (Shizuoka, Japan). *WB-W/W* and *WB-+/+* mice were obtained by mating of *WB-W/+* parents.

**Monoclonal Antibodies and Cell Staining.** Anti-B220 (RA3-6B2) (19), anti- $\mu^b$  (MB86) (20), Mac-1 (M1/70) (21), Gr-1 (granulocyte lineage marker, RB6-8C5) (22), anti-CD4 (GK1.5) (23), anti-CD8 (53-6.72) (24), and TER-119 (erythroid lineage marker) (25) were used. The hybridomas producing anti-*c-kit* mAbs, ACK2 (IgG2b) and ACK4 (IgG2a), were established from a rat immunized with IL-3-dependent normal mast cells as follows. IL-3-dependent mast cells were established from bone marrow cells of *WB-+/+* and *WB-W/W* neonates as described by Nakano et al. (26) except that we used recombinant IL-3 instead of PWM-stimulated spleen cell conditioned medium. Twenty million cultured mast cells derived from a normal littermate were injected intravenously twice into a Wistar rat and then spleen cells were prepared for the fusion with the X63.653.Ag8 (20). Antibody-producing hybridoma clones were selected on the basis of binding to normal but not *W/W* mouse-derived IL-3-dependent mast cells. Although these two mAbs recognize the extracellular domain of *c-kit*, ACK2 but not ACK4 can block blood cell formation in long-term bone marrow culture. All mAbs were used as the hybridoma supernatants or as the purified antibodies conjugated with FITC or allophycocyanin (APC) for cell staining. In the case of hybridoma supernatant, the stained cells were developed with the FITC-conjugated anti-rat *k* (MAR18.5) (27) as a second antibody. Cells were analyzed by Epics Profile (Coulter Electronics Inc., Hialeah, FL) or FACStar<sup>plus</sup>® (Becton-Dickinson Immunocytometry Systems, San Jose, CA) as described (28, 29).

**Antibody Injection.** B6 male mice were injected with 100  $\mu$ g or 1 mg purified antibodies intravenously every other day. Mice were then anesthetized and sacrificed by cervical dislocation, and bone marrow cells were analyzed for lineage markers and colony forming ability.

**Cytokines.** Murine recombinant IL-3, GM-CSF, and IL-7 were prepared and titrated as described previously (28, 30). 20 U/ml of IL-7, 200 U/ml of IL-3, 100 U/ml of GM-CSF, or 10% of L cell-conditioned medium as the source of M-CSF/CSF-1 (14) were used for the colony assay.

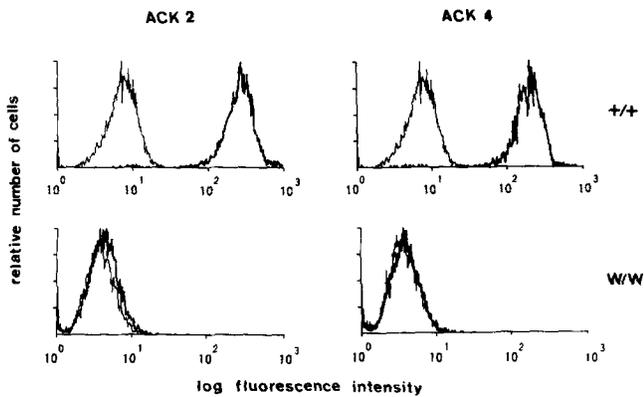
**Colony Assay.** Methylcellulose culture was performed as described (30). Briefly,  $2 \times 10^4$  bone marrow cells were incubated in 1 ml of culture medium containing  $\alpha$ -MEM (Gibco Laboratories, Grand Island, NY), 1.2% methylcellulose (Methocel A-4M; Muromachi Kagaku Kogyo, Tokyo, Japan), 30% FCS (HyClone, Lot No. 1115741; HyClone Laboratories Inc., Logan, UT), 1% deionized BSA (Sigma Chemical Co., St. Louis, MO), 50  $\mu$ M 2-ME and cytokines. On the seventh day of culture, aggregates consisting of >40 cells were scored as a colony.

**Colony-forming Unit-S (CFU-S) Assay.** Female B6 mice were X-irradiated (9 Gy). After 24 h, the mice were injected intravenously with  $5 \times 10^4$  or  $2.5 \times 10^5$  cells suspended in Eagle's MEM. Mice were anesthetized and sacrificed by cervical dislocation at 8 or 13 d after injection (31, 32). The spleens were removed and fixed in formalin/acetic acid/alcohol and colonies were counted. Controls injected with cell-free suspending medium had 2 colonies per 13 spleens.

**Depletion of *c-kit*<sup>+</sup> Cells from Bone Marrow Preparation.** Ten million bone marrow cells harvested from normal B6 mice were incubated with ACK4 or RA3-6B2, and resuspended in 1 ml of Eagle's MEM supplemented with 5% FCS. The paramagnetic microspheres coated with sheep anti-rat IgG (Dynabeads M-450; Dynal, Oslo, Norway) (33) were washed and suspended with same medium at the concentration of  $4 \times 10^8$  beads/ml. One ml of the beads suspension was mixed with the cell suspension prepared above and incubated on ice for 30 min with gentle shaking. The mixture was then diluted 1:5 by adding medium. The beads were removed twice by a magnetic concentrator (MPC1; Dynal) together with the cells bound to the beads. The cell recoveries were always around 30%. An aliquot of the remaining negative cells was stained again with the corresponding antibody and checked by flow cytometry. The remainder was tested in the colony assay described above.

## Results

**Establishment of Anti-*c-kit* Monoclonal Antibody.** IL-3-dependent mast cells derived from normal mouse bone marrow highly express the *c-kit* gene and are able to proliferate in the presence of the ligand of *c-kit* without the addition of IL-3 (5, 6, 10, 16). On the other hand, mast cells derived from *W/W* mice produce a *c-kit* molecule from which the transmembrane domain is deleted (4, 34). Consequently, the cells are expected to fail to express *c-kit* on the cell surfaces. This fact prompted us to make anti-*c-kit* mAbs by immunizing a rat with IL-3-dependent normal mast cells followed by selection on the basis of the ability to bind with the mast cells derived from a normal littermate mouse but not from a *W/W* mouse. By these criteria we obtained four independent mAbs against *c-kit*. Fig. 1 shows the surface staining of mast cells from both a normal and *W/W* mouse by two of the obtained mAbs, designated ACK2 and ACK4. These two mAbs recognized normal mast cells while they could not bind to mast cells derived from the *W/W* mouse. ACK2 recognized surface molecules whose mol wt were 120 and 160 kD as determined by Western blotting analysis of normal mast cells (data not shown). These sizes are in good accordance with those of *c-kit* (18). Furthermore, ACK2 bound to the COS7 cells transfected with the *c-kit* cDNA which was cloned into an expression vector while the parent cells were ACK2<sup>-</sup> (data not shown). These data indicate that the ACK mAbs specifically bind to the extracellular domain of *c-kit*.

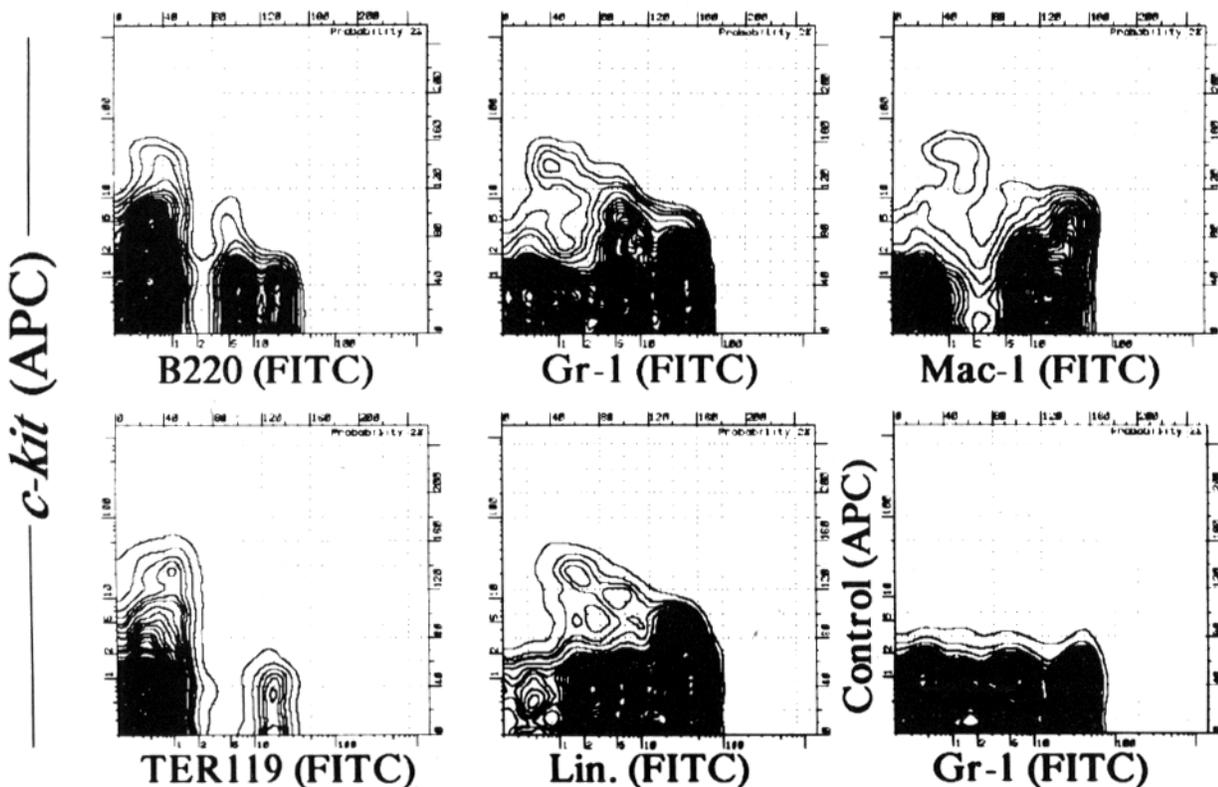


**Figure 1.** Surface staining of IL-3-dependent mast cells with anti-*c-kit* mAbs analyzed by flowcytometry. Mast cells were established from *W/W* and *+/+* littermates. Histograms of the cells stained by ACK2 and ACK4 are shown by the thick lines. The thin lines indicate the control staining by anti-CD4 mAb.

**Expression of *c-kit* in Bone Marrow.** By using the anti-*c-kit* mAb, ACK2, the expression of *c-kit* and other lineage markers in fresh bone marrow cells from adult C57BL/6 mouse was determined. As shown in Fig. 2, *c-kit* bright and dull populations coexist in bone marrow. The *c-kit* bright population

does not coexpress other lineage markers including B220, CD4, CD8, TER-119, Gr-1, nor Mac-1. This population accounts for  $3.3 \pm 0.3\%$  (mean  $\pm$  SD) of the total bone marrow cells. Most of the *c-kit* dull population coexpresses some of these lineage markers, although those which coexpress B220 or TER-119 are extremely low in number. The dull *c-kit* staining of Gr-1 and Mac-1 positive cells seems to be specific, since the addition of isotype matched unrelated mAb or normal rat serum does not affect this staining (data not shown). Thus, total *c-kit* positive cells account for  $7.8\% \pm 0.6\%$  of total bone marrow cells if the *c-kit* dull positive cells are included.

**Expression of *c-kit* on the Surface of Hemopoietic Progenitor Cells.** Next we investigated whether *c-kit* is expressed on the surface of hemopoietic progenitor cells including CFU-S. Normal bone marrow cells were incubated either with anti-*c-kit* mAb ACK4 or anti-B220 mAb 6B2, and antibody-bound cells were depleted by using anti-rat Ig antibody-coated magnetic beads. More than 90% of the positive cells were depleted by this treatment (data not shown). The number of CFC reactive to IL-3, GM-CSF, M-CSF, or IL-7 and spleen CFUs was measured (Table 1). When *c-kit*<sup>+</sup> cells were removed from bone marrow cells, CFU-IL-3, CFU-GM, CFU-M, and CFU-S were depleted almost completely, while CFU-IL-7 remained. Consistent with previous reports (30, 35), deple-



**Figure 2.** Expression of *c-kit* and lineage markers on B6 bone marrow cells. Cells were incubated with normal rat serum, then APC-conjugated anti-*c-kit* (ACK2) and FITC-conjugated anti-B220, Gr-1, Mac-1, or TER-119 antibody were added. A mixture of FITC-conjugated anti-B220, Gr-1, Mac-1, anti-CD4, and anti-CD8 antibodies were used to stain lineage markers (Lin.) positive cells. As a control, APC-conjugated mAbs against irrelevant antigens were used. Cells were washed, stained with propidium iodide (PI), and analyzed by flowcytometry. The dead cells stained with PI were gated out.

**Table 1.** Depletion of *c-kit*<sup>+</sup> Cells Eliminates Hemopoietic Progenitors from Bone Marrow of B6 Mice

Bone marrow cells*	No. of CFU/10 <sup>5</sup> cells†				
	CFU-S	CFU-IL-3	CFU-GM	CFU-M	CFU-IL-7
Control	18.0 ± 6.0	616.7 ± 44.3	403.4 ± 31.0	326.7 ± 15.2	93.3 ± 2.9
<i>c-kit</i> <sup>+</sup> -depleted	0.2 ± 0.2	15.0 ± 10.0	20.0 ± 8.7	3.3 ± 5.8	78.3 ± 11.5
B220 <sup>+</sup> -depleted	20.0 ± 5.5	818.4 ± 59.1	548.4 ± 34.5	476.7 ± 49.3	5.0 ± 5.0

\* The *c-kit*<sup>+</sup> or B220<sup>+</sup> cells were depleted from the bone marrow of B6 mouse by using antibody coated magnetic beads. For in vitro CFC assay, 2 × 10<sup>4</sup> cells were incubated in 1 ml of semisolid medium containing various cytokines for 7 d and aggregates consisting of 40 or more cells were scored as a colony. For the CFU-S assay, 5 × 10<sup>4</sup> cells from control or B220<sup>+</sup>-depleted sample, or 2.5 × 10<sup>5</sup> cells from ACK4<sup>+</sup>-depleted sample were injected into an irradiated mouse. The spleen was removed and fixed 8 d after injection and number of colonies was counted.

† Mean ± SD for triplicates.

tion of B220<sup>+</sup> cells removed CFU-IL-7 completely, while other CFCs and CFU-S remained intact. This result clearly indicates that *c-kit* is expressed on the surface of all hemopoietic progenitors, including CFU-IL-3, CFU-GM, CFU-M, and CFU-S, except CFU-IL-7 of the B cell lineage.

**Induction of Anemia by ACK2 Injection.** The results described in the preceding sections demonstrated the expression of *c-kit* on the surface of hemopoietic progenitor cells. However, it remained to be elucidated whether *c-kit* is functionally required for these progenitor cells in vivo. To address this question, we took advantage of an antagonistic anti-*c-kit* mAb, ACK2, which was shown to block the function of *c-kit* but not to be cytotoxic to *c-kit*<sup>+</sup> cells (46). If *c-kit* on the surface of hemopoietic progenitor cells is functioning in vivo, administration of ACK2 would induce severe anemia. In fact, ACK2 inhibited myelopoiesis in a long-term bone marrow culture with a stromal cell clone (our unpublished observation).

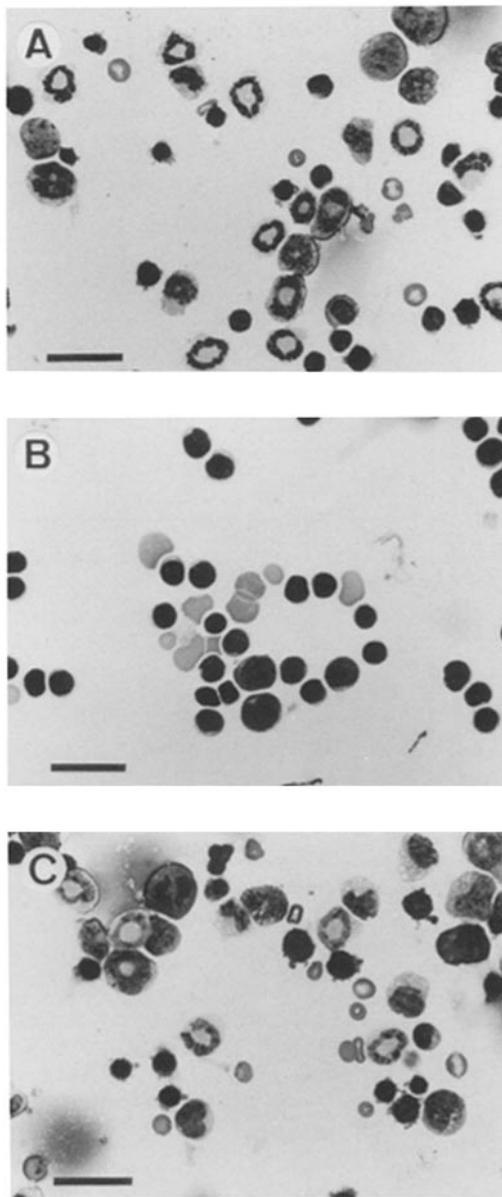
One milligram of purified ACK2 was injected into B6 mice intravenously every second day and the number of B220<sup>+</sup>, TER-119<sup>+</sup>, or Mac-1<sup>+</sup> cells in bone marrow was measured on various days after the initiation of ACK2 injection. Purified Mac-1 antibody was injected as a class matched control. Fig. 3 shows the morphology of the bone marrow cells from normal, Mac-1-injected, or ACK2-injected mice 12 d after the initial injection. No significant difference was observed between the bone marrow cells from normal and Mac-1 treated mice. In contrast, no polymorphonuclear cells or erythroblasts were present in the bone marrow of an ACK2 injected mouse, and most of the cells remaining in this bone marrow showed lymphoid morphology. This observation was confirmed by flow cytometry analysis (Fig. 4), which demonstrated that no Mac-1<sup>+</sup> cells nor TER-119<sup>+</sup> cells were present in the bone marrow of ACK2-injected mouse and that 90% of the total cells were B220<sup>+</sup> B lineage cells including 16% of surface IgM<sup>+</sup> cells. It is important to note that even after such a complete depletion of myeloid and erythroid cell lineages from bone marrow, the number of bone marrow cells recovered from ACK2-injected mice was nearly normal. This suggests that B lineage cells continued to grow in the ACK2-

treated mouse to fill the space from which myeloid and erythroid progenitor cells were purged.

Fig. 5 shows the time course of the changes in the content of each type of lineage marker positive cells after the initiation of ACK2 injection. The proportions of Mac-1<sup>+</sup> myeloid cells and B220<sup>+</sup> B lymphocytes did not change significantly by 4 d after the initiation of ACK2 injection. Mac-1<sup>+</sup> cells then decreased abruptly during the next 4 d. B220<sup>+</sup> cells increased in parallel with the decrease in Mac-1<sup>+</sup> cells. Although the number of total nucleated cells decreased to half the normal level during the initial 8 d along with the decrease in Mac-1<sup>+</sup> cells, it then returned to the control level by the overgrowth of B220<sup>+</sup> cells (data not shown). The number of TER-119<sup>+</sup> erythroid lineage cells decreased more rapidly than Mac-1<sup>+</sup> cells.

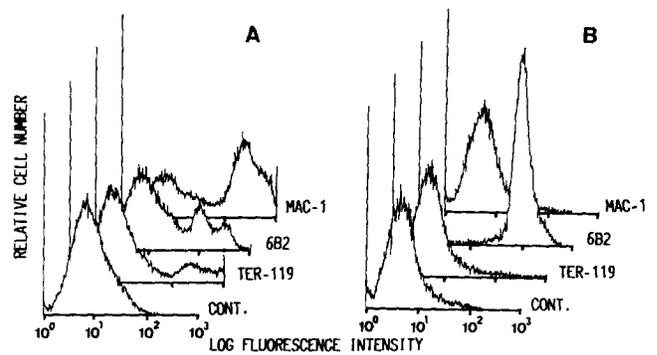
**Rapid Elimination of Hemopoietic Progenitor Cells by ACK2 Injection.** The results in the preceding section demonstrated that ACK2 injected into adult mouse could eliminate lineage marker positive mature myeloid and erythroid cells, while leaving the B lineage cell compartment intact. Next, we investigated the effect of ACK2 on the progenitor cells of myeloid and B cell lineages which can generate in vitro colonies in response to various hemopoietic growth factors. The same bone marrow cells from ACK2-treated mice used in the experiments in the previous section were analyzed for the presence of CFCs reactive to IL-3, GM-CSF, M-CSF, and IL-7. As shown in Fig. 6, CFU-IL3, CFU-GM, and CFU-M disappeared from the bone marrow much earlier than mature myeloid cells did, and a single ACK2 injection eliminated these CFCs almost completely. On the other hand, the number of CFU-IL-7 increased five- to six-fold, suggesting that active proliferation of B cell precursors was actually induced in ACK2-treated bone marrow. Because the addition of ACK2 did not affect the formation of colonies from normal bone marrow cells in semisolid medium containing various cytokines, the disappearance of CFC was not due to a cytostatic effect of ACK2 during the culture assay period (data not shown).

Furthermore, it is important to note that CFU-IL-3, CFU-GM, and CFU-M decreased simultaneously rather than in



**Figure 3.** Morphology of the cells in the bone marrow of the mice administered with antibodies. Cytospots were prepared from the bone marrow cells of a normal B6 mouse (A), a mouse given 6 shots of 1 mg ACK2 (B) or Mac-1 (C) on alternate days, and stained with May-Gruenwald-Giemsa solution. Scale bars indicate 20  $\mu\text{m}$ .

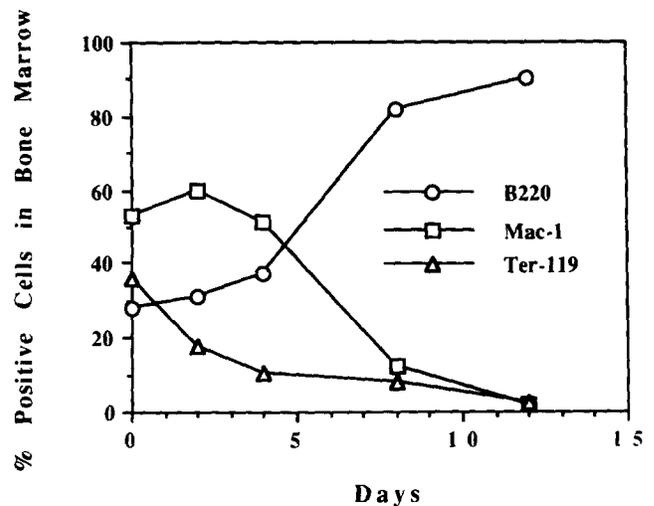
order of the proposed differentiation hierarchy of these progenitor cells (36, 37). This suggests that *c-kit* is functionally required for the maintenance of all of these CFCs in vivo. To further investigate whether or not each CFC has a different sensitivity to ACK2 treatment, the dose of ACK2 was decreased to 100  $\mu\text{g}$  and the numbers of CFCs were measured. The sensitivity of each CFC to ACK2 as estimated from Table 2 seemed not to differ significantly. Although only incomplete reduction of each CFC was induced by 100  $\mu\text{g}$  ACK2 in the bone marrow, the magnitude of reduction did not differ



**Figure 4.** Flow cytometric analysis of the bone marrow cells of an anti-*c-kit* antibody-treated mouse. Bone marrow cells harvested from a control mouse (A) or a mouse given 6 shots of 1 mg ACK2 (B) were stained with anti-B220, Mac-1, or TER-119. % positive cells estimated from the profiles are shown in Fig. 5 (Day 12).

significantly. We have also previously observed that 100  $\mu\text{g}$  is the dose at which adult spermatogenesis is completely blocked in vivo (Yoshinaga, K., S. Nishikawa, M. Ogawa, S.I. Hayashi, T. Kunisada, T. Fujimoto, and S.I. Nishikawa, manuscript submitted for publication). Thus, the sensitivities to ACK2 seem to vary among *c-kit*-dependent tissues.

**Elimination of Colony-forming Units-S (CFU-S) by ACK2 Injection.** Finally, we investigated whether the earliest hemopoietic progenitor cells which can give rise to spleen colonies upon transfer into lethally irradiated recipients are dependent on *c-kit* in vivo (38). Bone marrow cells were harvested from mice which had been given 6 i.v. shots of 1 mg mAb, and the number of CFU-S was determined (Table 3). Again, most of CFU-S were depleted from the bone marrow of ACK2-injected mouse irrespective of the day of the assay for spleen colony (31, 32). Interestingly, small but a significant number of day 13 CFU-S (approximately 10% of control) always re-



**Figure 5.** Effects of anti-*c-kit* antibody injection on the content of bone marrow cells. B6 mice were injected 1 mg ACK2 every other day and the content of each lineage marker<sup>+</sup> cells in bone marrow was analyzed as shown in Fig. 4.

**Table 2.** Effects of ACK2 Administration on Colony Forming Cells (CFCs) in the Bone Marrow of B6 Mice

Treatment*	No. of CFC/10 <sup>5</sup> cells†			
	CFU-IL-7	CFU-IL-3	CFU-GM	CFU-M
Control	188.3 ± 20.2	485.0 ± 34.6	358.3 ± 41.3	268.3 ± 29.3
ACK2 (0.1 mg)	335.0 ± 43.3	100.0 ± 25.9	125.0 ± 11.9	66.6 ± 8.1
ACK2 (1 mg)	228.3 ± 10.4	6.7 ± 2.9	16.7 ± 7.6	3.3 ± 5.8

\* B6 mice were given 2 shots of 0.1 or 1 mg ACK2 on alternate days. The number of CFCs in bone marrow was determined as described in the legend for Table 1.

† Mean ± SD for triplicates.

mained in the bone marrow, and these colonies were large mixed type colonies (Fig. 7). Therefore, a small fraction of day 13 CFU-S is resistant to the antagonistic activity of the anti-*c-kit* antibody.

### Discussion

The major aim of the present study was to characterize the *c-kit*<sup>+</sup> cells in adult mouse bone marrow and also to elucidate the functional role of this receptor tyrosine kinase molecule in this cell population. For this purpose, we used two mAbs against the extracellular domain of *c-kit*, ACK2 and ACK4. These two mAbs recognize different epitopes on the *c-kit* molecule, because binding of one antibody was not blocked by the other. Moreover, in vitro hemopoiesis on PA6 stromal cell clone (39, 40) or in Dexter's culture (41) was blocked completely by ACK2 but not by ACK4, suggesting that the former is antagonistic to *c-kit* function (our unpublished observation).

By using these mAbs, we demonstrated the presence of *c-kit*<sup>+</sup> cells in adult bone marrow. Interestingly, *c-kit*<sup>+</sup> cells were further subdivided into *c-kit*<sup>bright</sup> and *c-kit*<sup>dull</sup> populations, and *c-kit*<sup>bright</sup> cells did not coexpress other lineage

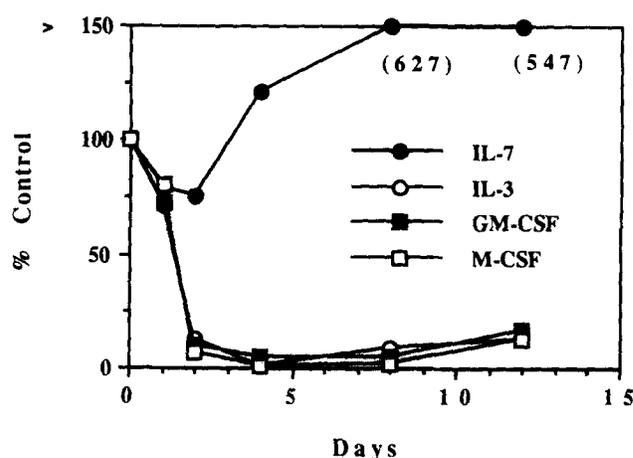
**Table 3.** Effects of Antibody Administration on Colony-forming Unit-S (CFU-S) in the Bone Marrow of B6 Mice

Antibody*	No. of CFU-S/10 <sup>5</sup> cells†	
	Day 8	Day 13
Control	19.3 ± 3.1	11.7 ± 2.3
ACK2	0.4 ± 0.4	1.5 ± 0.4
Mac-1	20.0 ± 6.0	ND <sup>§</sup>

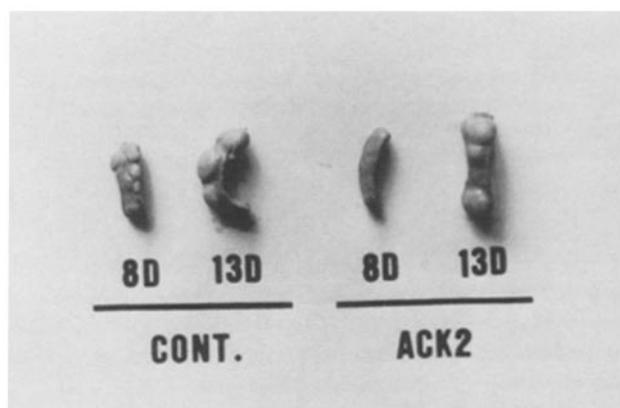
\* B6 mice were given 6 shots of 1 mg antibodies on every other day. 50,000 bone marrow cells from the control mouse or Mac-1 treated mouse, or 2.5 × 10<sup>5</sup> bone marrow cells of ACK2 treated mouse were injected into irradiated recipients. The spleens were removed and fixed 8 or 13 d after the injection and the number of colonies was counted.

† Mean ± SD for quadruplicates.

§ Not done.



**Figure 6.** Effects of anti-*c-kit* antibody injection on the number of CFCs responding to various cytokines in bone marrow. The same cells shown in Fig. 5 were analyzed for the number of CFU-IL-7, CFU-IL-3, CFU-GM, and CFU-M. The number of CFU-IL-7 at day 8 and day 12 (% control) are shown in parentheses since the values are out of scale.



**Figure 7.** Elimination of CFU-S from the bone marrow of an anti-*c-kit* antibody-injected mouse. The bone marrow cells of the B6 mouse given 6 shots of 1 mg ACK2 on alternate days were analyzed for the number of CFU-S as described in the legend for Table 3. Note that five-fold larger number of cells was injected into the recipients in the case of the ACK2-treated mouse.

markers while most of the *c-kit*<sup>dull</sup> population expressed myeloid markers such as Gr-1 and Mac-1. Probably, a small fraction of *c-kit*<sup>dull</sup> cells coexpressed B220, but no TER-119<sup>+</sup> cells were included in this population. This staining pattern indicates that *c-kit* is highly expressed in the lineage marker negative (*lin*<sup>-</sup>) immature cell population and that *c-kit* expression decreases upon maturation into *lin*<sup>+</sup> cells, although low level expression continues for some time. Since Müller-Sieburg et al. have shown that all CFCs were present in the *lin*<sup>-</sup> population (42), *c-kit*<sup>dull</sup>, *lin*<sup>+</sup> population might have lost the progenitor activity. To confirm this, we are currently trying to sort each population to estimate the stem cell activity. In fact, a recent preliminary result suggests that CFU-S was highly enriched in a *c-kit*<sup>bright</sup>, Thy-1<sup>dull</sup>, *lin*<sup>-</sup> population (Okada, S., H. Nakauchi, K. Nagayoshi, S. Nishikawa, S.I. Nishikawa, Y. Miura, and T. Suda, manuscript submitted for publication). Our present result clearly indicated that almost all hemopoietic progenitor cells including CFCs reactive to IL-3, GM-CSF, or M-CSF and also CFU-S expressed *c-kit* on the cell surface, but B lineage progenitors which form colonies in response to IL-7 were *c-kit*<sup>-</sup>. Thus, we expect that *c-kit* expression would be additional and powerful markers in order to purify hemopoietic stem cells in various differentiation stages.

Since most of hemopoietic progenitors expressed *c-kit*, the next issue to be resolved was whether or not *c-kit* on the surface of these hemopoietic progenitors is functioning in vivo. The fact that the *W/W* homozygous mouse has no constitutive hemopoiesis in postnatal life indicates that *c-kit* is functioning at least in some progenitor cells (15). Previously, it was reported that CFCs do exist in the *W/W<sup>v</sup>* mouse while CFU-S is absent (43). This may well suggest that *c-kit* is functionally required only for the most immature progenitors, although it is expressed in both CFC and CFU-S. To address this question, we took advantage of a mAb capable of antagonistically blocking the function of *c-kit*. Injection of this antibody resulted in a complete and simultaneous elimination of all hemopoietic progenitor cells including CFU-S and CFCs reactive to IL-3, GM-CSF or even M-CSF which has been considered to be a growth factor for the precursor restricted to the macrophage lineage. It could be that ACK2 is cytotoxic antibody eliminating all *c-kit*<sup>+</sup> cells, thereby depleting all types of hemopoietic progenitors that express *c-kit*. We think, however, that this possibility is unlikely for the following reasons. First, although peritoneal mast cells highly express *c-kit*, i.p. injection of ACK2 does not affect the number of mast cells in the peritoneal cavity (our unpublished observation). Second, although all melanocytes in the adult hair follicle express *c-kit*, only those in the activated hair follicles are affected by ACK2 injection, suggesting that only *c-kit* function is blocked by this treatment (46). Third, although both spermatogonia and oocytes express *c-kit*, ACK2 injection blocked only spermatogenesis. Fourth, although Mac-1 used as a class matched control antibody also binds to myeloid cells, hemopoiesis in Mac-1-treated mice was normal. Lastly, rat mAbs of IgG2b class against various cell surface molecules have been used as antagonistic blockers of molecular interaction on the cell surface, and all previous results

unequivocally indicated that rat-IgG2b mAb was cytostatic rather than cytotoxic (44).

It could also be that ACK2 enhances the differentiation of the progenitor cells, thereby resulting in a depletion of stem cells. We think this possibility is also unlikely, because overgrowth of lineage marker bearing mature cells was never detected at any point after the injection of ACK2. Furthermore, despite extensive trials, we failed to detect any ligand mimicking activity of ACK2 to induce in vitro differentiation or proliferation of hemopoietic progenitors. On the other hand, the recombinant ligand for *c-kit* has recently been reported to have obvious in vitro activity to induce the proliferation of various hemopoietic precursor cells (5, 9–11).

Consequently, it is very likely that ACK2 acts as an antagonistic antibody to *c-kit* function, although it remains to be elucidated whether ACK2 directly prevents the binding of the ligand to *c-kit*. Given that this is the case, our result must be interpreted as indicating that maintenance of hemopoietic progenitors in adult bone marrow is dependent on *c-kit* and its ligand. Because recent studies demonstrated that a recombinant ligand for *c-kit* induced colonies consisting of multiple cell lineages (9–11), the abolition of all CFCs from ACK2-treated bone marrow may be due to the blockade of recruitment of lineage restricted progenitors from multipotent stem cells. However, it is important to note that all CFCs from those reactive to multi-CSF to even M-CSF disappeared from bone marrow simultaneously. If *c-kit* is functionally required for the maintenance of multipotent hemopoietic progenitor cells giving rise to lineage restricted progenitor cells such as CFU-M, there must be a time lag between disappearance of progenitors reactive to multi-CSF and to M-CSF. In this context, it is interesting that the ligand for *c-kit* is able to cooperate with any of other hemopoietic growth factors and enhances the number and size of colonies induced in bone marrow cells (10, 11). These results in conjunction with our present result imply that the maintenance of both multipotent and lineage restricted hemopoietic progenitor cells is in fact dependent on *c-kit*, although some other factors may also be required.

In contrast to the *c-kit* dependency of myeloid and erythroid progenitors, B lymphopoiesis was not significantly affected by ACK2 injection. More interestingly, a subset of B lineage cells did overgrow to fill the bone marrow space from which other cell lineages were purged. On the other hand, it was demonstrated that the ligand for *c-kit* also remarkably enhanced the colony formation of B cell precursors in response to IL-7 (11), suggesting the role of *c-kit* in intramarrow B lymphopoiesis. This result, however, is not inconsistent with our present result, because the previous studies clearly demonstrated that there is a subpopulation of B lineage cells which proliferates in response to IL-7 alone (28, 30, 35, 45). Therefore, the population capable of proliferating in response to IL-7 alone could be the subset that overproliferated in the ACK2-treated mouse. Alternatively, it could be that there is another subset of B lineage precursors which requires yet unidentified signals for proliferation. Since the ligand for *c-kit*, and ACK2 which specifically inhibits the *c-kit* function are now available, this issue will be resolved soon.

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