Expression and Function of c-kit in Hemopoietic Progenitor Cells

By Minetaro Ogawa,* Yumi Matsuzaki,† Satomi Nishikawa,* Shin-Ichi Hayashi,* Takahiro K uninada,† Tetsuo Sudo,§ Tatsuo Kina,‖ Hiromitsu Nakauchi,§ and Shin-Ichi Nishikawa*

From the *Department of Pathology, Institute for Medical Immunology, Kumamoto University Medical School, Kumamoto, Kumamoto 860, Japan; the †Laboratory of Molecular Regulation of Aging, Frontier Research Program, The Institute of Physical and Chemical Research (RIKEN), Tsukuba, Ibaraki 305, Japan; the ‡Biomaterial Research Institute Co. Ltd., Yokohama, Kanagawa 244, Japan; and the †Department of Molecular Pathology, Chest Disease Research Institute, Kyoto University, Kyoto, Kyoto 606, Japan

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+ 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+ 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)1/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
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-W/+ mice were obtained by mating of WB-W/+ parents.

Monoclonal Antibodies and Cell Staining. Anti-B220 (RA3-6B2) (19), anti-αM (MB66) (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 IL3-dependent normal mast cells as follows. IL3-dependent mast cells were established from bone marrow cells of WB-W/+ and WB-W/W neonates as described by Nakano et al. (26) except that we used recombinant IL3 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 IL3-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 FACStarPlus (Becton-Dickinson Immunocytometry Systems, San Jose, CA) as described (28, 29).

Antibody Injection. B6 male mice were injected with 100 μg 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 IL3, 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 x 10^5 bone marrow cells were incubated in 1 ml of culture medium containing α-MEM (Gibco Laboratories, Grand Island, NY), 1.2% methylcellulose (Methocel A-4M; Muromachi Kagaku Kogyo, Tokyo, Japan), 30% FCS (HyClone, Lot No. 1115744; HyClone Laboratories Inc., Logan, UT), 1% deionized BSA (Sigma Chemical Co., St. Louis, MO), 50 μM 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 x 10^6 or 2.5 x 10^6 cells suspended in Eagle's MEM. Mice were anesthetized and sacrificed by cervical dislocation 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+ 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 x 10^9 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. IL3-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 IL3 (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 IL3-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. By these criteria we obtained four independent mAbs against c-kit. 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-negative (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 about 3.3 ± 0.3% (mean ± SD) of the total bone marrow cells. Most of the c-kit dull population coexpress some of these lineages 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% ± 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 CFU reactive to IL-3, GM-CSF, M-CSF, or IL-7 and spleen CFUs was measured (Table 1). When c-kit+ 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.
tion of B220+ 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 hematopoietic 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 hematopoietic 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+ cells (46). If c-kit on the surface of hematopoietic 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+ , TER-119+, or Mac-1+ 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+ cells nor TER-119+ cells were present in the bone marrow of ACK2-injected mouse and that 90% of the total cells were B220+ B lineage cells including 16% of surface IgM+ 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+ myeloid cells and B220+ B lymphocytes did not change significantly by 4 d after the initiation of ACK2 injection. Mac-1+ cells then decreased abruptly during the next 4 d. B220+ cells increased in parallel with the decrease in Mac-1+ 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+ cells, it then returned to the control level by the overgrowth of B220+ cells (data not shown). The number of TER-119+ erythroid lineage cells decreased more rapidly than Mac-1+ cells.

**Rapid Elimination of Hematopoietic 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 hematopoietic 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-IL-3, 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

---

**Table 1. Depletion of c-kit+ Cells Eliminates Hematopoietic Progenitors from Bone Marrow of B6 Mice**

| Bone marrow 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 |
| c-kit−-depleted   | 0.2 ± 0.2 | 15.0 ± 10.0 | 20.0 ± 8.7 | 3.3 ± 5.8 | 78.3 ± 11.5 |
| B220−-depleted    | 20.0 ± 5.5 | 818.4 ± 59.1 | 548.4 ± 34.5 | 476.7 ± 49.3 | 5.0 ± 5.0 |

* The c-kit+ or B220+ cells were depleted from the bone marrow of B6 mouse by using antibody coated magnetic beads. For in vitro CFC assay, 2 × 10⁴ 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 × 10⁴ cells from control or B220−-depleted sample, or 2.5 × 10⁴ cells from ACK2−-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.
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 μg 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 μg ACK2 in the bone marrow, the magnitude of reduction did not differ significantly. We have also previously observed that 100 μg 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 hematopoietic 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-
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+ 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+ cells in adult bone marrow. Interestingly, c-kit+ cells were further subdivided into c-kitbright and c-kitdim populations, and c-kitbright 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 | 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$^4$    |

* 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 × 10⁶ 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.

$^4$ Not done.
markers while most of the c-kitd11 population expressed myeloid markers such as Gr-1 and Mac-1. Probably, a small fraction of c-kitd11 cells coexpressed B220, but no TER-119+ cells were included in this population. This staining pattern indicates that c-kit is highly expressed in the lineage marker negative (lin-) immature cell population and that c-kit expression decreases upon maturation into lin+ cells, although low level expression continues for some time. Since Müller-Sieburg et al. have shown that all CFCs were present in the lin- population (42), c-kitd11, lin- 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-kitbright, Thy-1dull, lin- 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 lineage progenitors which form colonies in response to IL-7 were c-kit-. 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>+</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+ 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.
References

1. Qiu, F., P. Ray, K. Brown, P.E. Barker, S. Jhanwar, F.H. Ruddle, and P. Besmer. 1988. Primary structures of c-kit: relationship with the CSF-1/PDGF receptor kinase family. Oncogene:235:335:88.
2. Chabot, B., D.A. Stephenson, V.M. Chapman, P. Besmer, and A. Bernstein. 1988. The proto-oncogene c-kit encoding a trans-membrane tyrosine kinase receptor maps to the mouse W locus. Nature (Lond.) 335:88.
3. Geissler, E.N., M.A. Ryan, and D.E. Housman. 1988. The dominant-white spotting (W) locus of the mouse encodes the c-kit proto-oncogene. Cell. 55:185.
4. Nocka, K., J.C. Tan, E. Chiu, T.Y. Chu, P. Ray, P. Traktman, and P. Besmer. 1990. Molecular bases of dominant negative and loss of function mutations at the murine c-kit/white spotting locus: W^y, W^y, W^u and W. EMBO (Eur. Mol. Biol. Organ.) J. 9:1805.
5. Nocka, K., J. Buck, E. Levi, and P. Besmer. 1990. Candidate ligand for the c-kit transmembrane kinase receptor: KL, a fibroblast derived growth factor stimulates mast cells and erythroid progenitors. EMBO (Eur. Mol. Biol. Organ.) J. 9:3,287.
6. Williams, D.E., J. Eisenman, A. Baird, C. Rauch, K.V. Ness, C.J. March, I.S. Park, U. Martin, D.Y. Mochizuki, H.S. Boswell, G.S. Burgess, D. Cosman, and S.D. Lyman. 1990. Identification of a ligand for the c-kit proto-oncogene. Cell. 63:167.
7. Zsebo, K.M., D.A. Williams, E.N. Geissler, V.C. Broudy, F.H. Martin, H.L. Atkin, R.Y. Hsu, N.C. Birkett, K.H. Okino, D.C. Murdock, F.W. Jacobsen, K.E. Langley, K.A. Smith, T. Takeishi, B.M. Cattanach, S.J. Galli, and S.V. Suggs. 1990. Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for c-kit tyrosine kinase receptor. Cell. 63:213.
8. Huang, E., K. Nocka, D.R. Beier, T-Y. Chu, J. Buck, H-W. Lahm, D. Wellner, P. Leder, and P. Betser. 1990. The hematopoietic growth factor KL is encoded by the Sl locus and is the ligand of the c-kit receptor, the gene product of the W locus. Cell. 63:225.
9. Anderson, D.M., S.D. Lyman, A. Baird, J.M. Wignall, J. Eisenman, C. Rauch, C.J. March, H.S. Boswell, S.D. Gimpel, D. Cosman, and D.E. Williams. 1990. Molecular cloning of mast cell growth factor, a hematopoietin that is active in both membrane bound and soluble forms. Cell. 63:235.
10. Zsebo, K.M., J. Wypych, I.K. McNiece, H.S. Lu, K.A. Smith, S.B. Karkare, R.K. Sachdev, V.N. Yuschenskoff, N.C. Birkett, L.R. Williams, V.N. Satyagal, W. Tsung, R.A. Bosselman, E.A. Mendiaz, and K.E. Langley. 1990. Identification, purification, and biological characterization of hematopoietic stem cell factor from buffalo rat liver-conditioned medium. Cell. 63:195.
11. Martin, F.H., S.V. Suggs, K.E. Langley, H.S. Lu, J. Ting, K.H. Okino, C.F. Morris, I.K. McNiece, F.W. Jacobsen, E.A. Mendiaz, N.C. Birkett, K.A. Smith, M.J. Johnson, V.P. Parker, J.C. Flores, A.C. Patel, E.F. Fisher, H.O. Erjavec, C.J. Herrera, J. Wypych, R.K. Sachdev, J.A. Pope, I. Leslie, D. Wen, C-H. Lin, R.L. Cupples, and K.M. Zsebo. 1990. Primary structure and functional expression of rat and human stem cell factor DNAs. Cell. 63:203.
12. Copeland, N.G., D.J. Gilbert, B.C. Cho, P.J. Donovan, N.A. Jenkins, D. Cosman, D. Anderson, S.D. Lyman, and D.E. Williams. 1990. Mast cell growth factor maps near the steel locus on mouse chromosome 10 and is deleted in a number of steel alleles. Cell. 63:175.
13. Flanagan, J.G., and P. Leder. 1990. The kit ligand: a cell surface molecule altered in steel mutant fibroblasts. Cell. 63:185.
14. Yoshida, H., S-I. Hayashi, T. Kunisada, M. Ogawa, S. Nishikawa, H. Okamura, T. Sudo, L.D. Shultz, and S-I. Nishikawa. 1990. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature (Lond.) 345:442.
15. Russell, E.S. 1979. Hereditary anemia of the mouse: a review for geneticists. Adv. Genet. 20:357.
16. Kitamura, Y. 1989. Heterogeneity of mast cells and phenotypic change between subpopulations. Annu. Rev. Immunol. 7:59.
17. Tan, J.C., K. Nocka, P. Ray, P. Traktman, and P. Besmer. 1990. The dominant W^y spotting phenotype results from a missense mutation in the c-kit receptor kinase. Science (Wash. DC). 247:209.
18. Nocka, K., S. Majumder, B. Chabot, P. Ray, M. Cervone, A. Bernstein, and P. Besmer. 1989. Expression of c-kit gene products in known cellular targets of W mutations in normal and W mutant mice-evidence for an impaired c-kit in mutant mice. Genes & Dev. 3:816.
19. Coffman, R.L. 1986. Surface antigen expression and immunoglobulin gene rearrangement during mouse pre-B cell development. Immunol. Rev. 69:5.
20. Nishikawa, S-I., Y. Sasaki, T. Kina, T. Amagai, and Y. Katamura. 1986. A monoclonal antibody against Igh6-4 determinant. Immunogenetics. 23:137.
21. Springer, T., G. Galfrè, D.S. Secher, and C. Milstein. 1979. Mac-l: a macrophage differentiation antigen identified by monoclonal antibody. Eur. J. Immunol. 9:301.
22. Spangrude, G.J., S. Heimfeld, and I.L. Weissman. 1988. Purification and characterization of mouse hematopoietic stem cells. Science (Wash. DC). 241:58.
23. Dialynas, D.P., Z.S. Quan, K.A. Wall, A. Pierres, J. Quintans,
M.R. Loken, M. Pierres, and F.W. Fitch. 1983. Characterization of the murine T cell surface molecule, designated L3T4, identified by the monoclonal antibody GK1.5: similarity of L3T4 to the human Leu-3/T4 molecule. J. Immunol. 131:2,445.

24. Ledbetter, J.A., and L.A. Herzenberg. 1979. Xenogenic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol. Rev. 47:63.

25. Ikuta, K., T. Kanakura, H. Asai, and Y. Kitamura. 1990. A developmentswitch in thymic lymphocytematuration potential occurs at the level of hematopoietic stem cells. Cell. 62:863.

26. Nakano, T., Y. Kanakura, H. Asai, and Y. Kitamura. 1987. Changing processes from bonemarrow-derived cultured mast cellstothectissue-type mast cells in theperitoneal cavity of mast cell deficient W/W^v mice: association of proliferation arrest and differentiation. J. Immunol. 138:544.

27. Lanier, L.L., G.A. Gutman, D.E. Lewis, S.T. Griswold, and N.L. Warner. 1982. Monoclonal antibodies against rat immunglobulin chains. Hybridoma. 1:125.

28. Hayashi, S.-I., T Kunisada, M. Ogawa, T. Sudo, H. Kodama, T. Suda, S. Nishikawa, and S.-I. Nishikawa. 1991. Exon skipping by mutation of an authentic splice site of c-kit gene in W/W^v mouse. Nucleic Acids Res. 19:1267.

35. Lee, G., A.E. Namen, S. Gillis, L.R. Ellingsworth, and P.W. Kincade. 1989. Normal B cell precursors responsive to recombinant murine IL-7 and inhibition of IL-7 activity by transforming growth factor-β. J. Immunol. 142:3,875.

39. Kodama, H., Y. Amagai, H. Koyama, and S. Kasai. 1982. A new preadipose cell line derived from newborn mouse calvaria can promote the proliferation of pluripotent hematopoietic stem cells in vitro. J. Cell. Physiol. 112:89.

40. Nishikawa, S.-I., M. Ogawa, S. Nishikawa, T. Kunisada, and H. Kodama. 1988. B lymphopoiesis on stromal cell clone: stromal cell clones acting on different stages of B cell differentiation. Eur. J. Immunol. 18:1,767.

41. Dexter, T.M., T.D. Allen, and L.G. Lajtha. 1977. Conditions controlling the proliferation of hematopoietic stem cells in vitro. J. Cell. Physiol. 91:335.

42. Müller-Sieburg, C.E., K. Townsend, I.L. Weissman, and D. Rennick. 1988. Proliferation and differentiation of highly enriched mouse hematopoietic stem cells and progenitor cells in response to defined growth factors. J. Exp. Med. 167:1,825.

43. Harrison, D.E., and E.S. Russell. 1972. The response of W/W^v and S/S^v anaemic mice to haemopoietic stimuli. Br. J. Haematol. 22:155.

44. Zufzufa-Pfllcker, J.C., S.A. McCarthy, M. Weston, D.L. Longo, A. Singer, and A.M. Kruisbeek. 1989. Role of CD4 in thymocyte selection and maturation. J. Exp. Med. 169:2,085.

45. Sudo, T., M. Ito, Y. Ogawa, M. Izuka, H. Kodama, T. Kunisada, S.-I. Hayashi, M. Ogawa, K. Sakai, S. Nishikawa, and S.-I. Nishikawa. 1989. Interleukin 7 production and function in stromal cell-dependent B cell development. J. Exp. Med. 170:333.

46. Nishikawa, S., M. Kusakabe, K. Yoshinaga, M. Ogawa, S.I. Hayashi, T. Kunisada, T. Era, T. Sakakura, and S.-I. Nishikawa. In vitro manipulation of coat color formation by a monoclonal anti-c-kit antibody: two distinct waves of c-kit dependency during melanocyte development. EMBO (Eur. Mol. Biol. Organ.) J. In press.