LIFE SPAN OF MULTIPOTENTIAL HEMATOPOIETIC STEM CELLS IN VIVO

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The hematopoietic system is maintained throughout adult life by a population of immature precursors known as the multipotential stem cells. These stem cells are unique within the system in that they have the capacity to generate progeny of all the blood cell lineages, as well as the capacity to generate cells with a potential similar to their own, a characteristic often referred to as self-renewal (1–8). It is this self-renewal capacity that enables stem cells to give rise to functional blood cells over long periods of time in an unperturbed hematopoietic system or to generate a new blood cell system when transplanted to hematopoietically deficient recipients.

Although these characteristics of stem cells have been recognized for some time, it is still not known to what extent stem cells can self-renew. If this capacity is unlimited, then stem cells should be "immortal" and able to function for an entire lifetime. If this is true, then the clonal make-up of the hematopoietic system should remain relatively stable over extended periods of time. On the other hand, if the self-renewal capacity of the stem cell is limited, then these cells would have a finite life span. Under this latter condition only a portion of the total stem cell pool would be active at a given time, and new stem cells would be drawn from this pool to replace those that are dying. This constant turnover in the active stem cell population would lead to a change, with time, in the clonal make-up of the different hematopoietic lineages. Hematopoiesis then would be maintained by a succession of short-lived clones as was proposed by the clonal succession model of Kay (9). A number of studies (6, 8, 10, 11) have demonstrated that the hematopoietic system of an irradiated reconstituted mouse does in fact undergo some clonal changes with time and thus would support the concept of short-lived stem cells. Experiments from several other groups, however, have provided evidence indicating that at least a subpopulation of stem cells are long-lived, able to function for significant periods of time (7, 12–14).

One approach that has been used to address this issue is to uniquely mark stem cells through the use of retroviral integration sites and then follow the fate of their progeny when introduced into recipient mice (6, 7, 11). In a previous report (7) we analyzed the clonal make-up of the various hematopoietic lineages of such recipients

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at different points in time and were able to demonstrate the presence of clones that persisted for a minimum of 5 mo, as well as the presence of clones that underwent changes during this period of time. In this report we extend these earlier findings and demonstrate for the first time the presence of a population of primitive multipotential stem cells that can function for at least 15 mo after reconstitution. In addition, we provide evidence that suggests that this primitive stem cell population can clonally expand during the regeneration of a new hematopoietic system.

Materials and Methods

Mice. 12-wk-old CBA/Ca mice (Banton and Kingman, Humberside, England) were used as both recipients and donors. Recipients received 950 rad of irradiation before reconstitution.

Bone Marrow Infection and Reconstitution. The bone marrow infection and reconstitution was carried out essentially as described previously (4, 7). Recipient mice were grafted with day 17 embryonic thymuses under each kidney capsule. 3 wk later these mice were irradiated and reconstituted with bone marrow cells that had been infected with the N2 recombinant retroviruses (4). Infection was achieved by co-culturing bone marrow from 5-fluorouracil (5-FU)[1]-treated mice with the virus-producing cells in cultures consisting of Iscove's modified Dulbecco's medium (IMDM), FCS (5%), WEHI-3-conditioned medium (CM) (20%) as a source of IL-3, and CM from a bone marrow-derived stromal cell line, 95/1.7 (5%), as a source of accessory growth factors (15). 18 h later the bone marrow cells were harvested from the virus-producing cells and put into a selection culture that consisted of IMDM, horse serum (20%), hydrocortisone (10^{-6} M), WEHI-3 CM (20%), 95/1.7 CM (5%), and G418 (1 mg/ml, active substance). 2 d later these cells were harvested and used for reconstitution. Using this protocol, >85% of the in vitro colony-forming cells within this population were G418 resistant ($G418^R$). The marrow from the femurs and tibias of six donors was used to reconstitute each recipient. Due to losses during drug selection, this represented $\sim 2 \times 10^6$ cells.

Preparation of Cells from Individual Lineages. 2 mo after reconstitution half of the grafted thymuses and half of the spleen were removed from the recipient mice. DNA was prepared from the grafted thymuses. Each spleen sample was subsequently divided into four portions. One quarter of this sample was used to prepare DNA and is designated as "SPL." A second part of the spleen sample was cultured in the presence of Con A (5 μg/ml) and IL-2 for 5–7 d to enrich for T lymphocytes and is referred to as "Con A." A third portion was treated with anti-Thy-1.2 antibody and complement to yield a "non-T" population enriched for B cells. Dead cells were removed from each of the Con A and non-T populations by Lympholyte (Cedarlane Laboratories, Ontario, Canada) density gradient centrifugation. More than 95% of the Con A, and <5% of the non-T populations were found to express the Thy-1 antigen. In contrast, <5% of the Con A population and >70% of the non-T population were surface Ig^+. The fourth portion of the splenic sample was cultured in the presence of WEHI-3 CM (IL-3) for 3–4 wk. At the end of this culture period >90% of the cells had the morphology of mast cells following May-Grünwald-Giemsa staining. DNA prepared from this population is referred to as "MAST."

7 mo after reconstitution, four recipients (mice nos. 30, 31, 33, and 35) received an injection of 5-FU. It is known that this treatment depletes the marrow of many intermediate stage cycling precursors (16, 17), and therefore could activate any "silent" stem cells. Two of these recipients, mice 30 and 33, died several weeks after this injection, presumably from the toxic effects of the 5-FU.

At 10 mo after reconstitution the primary recipients were sacrificed and the remainder of the spleen, the bone marrow (BM), the lymph nodes (LN), and the endogenous thymus (THY-E), were removed. The spleen was fractionated as described above, and the BM was

[1] *Abbreviations used in this paper:* BM, bone marrow; CM, conditioned medium; 5-FU, 5-fluorouracil; IMDM, Iscove's modified Dulbecco's medium.

separated into four portions. One portion was used for DNA preparation. A second portion was used for growing mast cells as described above. Macrophages were grown from a third portion of the bone marrow by culturing the cells in IMDM that contained WEHI-3 CM (10%) and L cell CM (35%) as a source of CSF-1 (18). 48 h later, the nonadherent cells that contained the macrophage precursors were removed and replated in cultures that contained IMDM and L cell-CM (35%). 10 d later these cells were harvested and used as a population highly enriched for macrophages. The fourth portion of the BM (10^7 cells) was used to reconstitute two secondary irradiated recipients (5×10^6 cells per mouse). 7 mo after reconstitution the secondary recipients were killed and cell populations prepared as described above. DNA was prepared from all of the organs and from the isolated cell populations from the various recipients. At the time mice were killed the percentage of in vitro colony forming cells that were resistant to G418 (1 mg/ml active substance) was determined as previously described (4). There was no significant difference in the frequency of resistance between the various types of colonies; therefore, only the data for total colonies are given. In total, the progeny of the infected stem cells were analyzed in the recipients at three different time points: 2, 10, and 17 mo following the initial reconstitution. The primary recipients are designated as mice 31, 34, 35, and 36 and the secondary recipients as mice 31*A, 31*B, 34*, 35*A, and 35*B.

Southern Blot Analysis. High molecular weight DNA (10 μg) was digested with either Hind III or Bam HI restriction enzymes that cut outside the provirus, and was analyzed by the alkaline transfer procedure (19). The resulting blots were hybridized with a nick translated probe derived from the *neo* gene in the virus.

Results

Clonal Make-Up of the Primary Recipients. The DNA from the various hematopoietic tissues and cell lineages from three of the four primary recipients (mice 31, 35, and 36) contained many fragments that hybridize to the neo probe, indicating the presence of many clones (Figs. 1-3). In contrast the tissues from mouse 34 contained only three predominant clones at the time this animal was killed (Fig. 3). The conditions under which these experiments were performed allow the detection of only those clones that represent at least 5% of the total cells. The percentage of the hematopoietic system that carries the N2 provirus is analyzed by determining the frequency of G418-resistant colony-forming cells. In these studies this ranged between 10 and 82% (see figure legends). This is a minimal estimate of the frequency of retroviral marked cells due to the fact that many N2-infected cells do not express sufficient levels of the neo^R gene to be resistant to the level of G418 used in these assays (4). By comparing the clonal make-up of the tissues and cell populations at 2 mo to that at 10 mo, it is possible to identify clones in each of the primary recipients that persisted throughout this part of the experiment. Examples of these persistent clones are marked "P" in Figs. 1-3. These persistent clones were also found in those animals that received an injection of 5-FU. For example, in mouse 31 (Fig. 1) the same clones can be identified in the total spleen cell population and the non-T portion of the spleen at both time points, as well as in the splenic-derived mast cells at the first time point and the BM-derived mast cells at the second time point. In mouse 35 (Fig. 2) a number of the clones found in the spleen and the non-T portion of the spleen at the first time point can also be identified in the BM, the BM-derived mast cells and the lymph node at 10 mo after reconstitution (Fig. 2). The persistent clones found in all of these animals tended to be present in both myeloid and lymphoid tissues, indicating that they were derived from multipotential stem cells. These findings demonstrate that the stem cells that gave rise to these clones could function for a minimum of 8 mo, the time between the two assay points. In addition to these

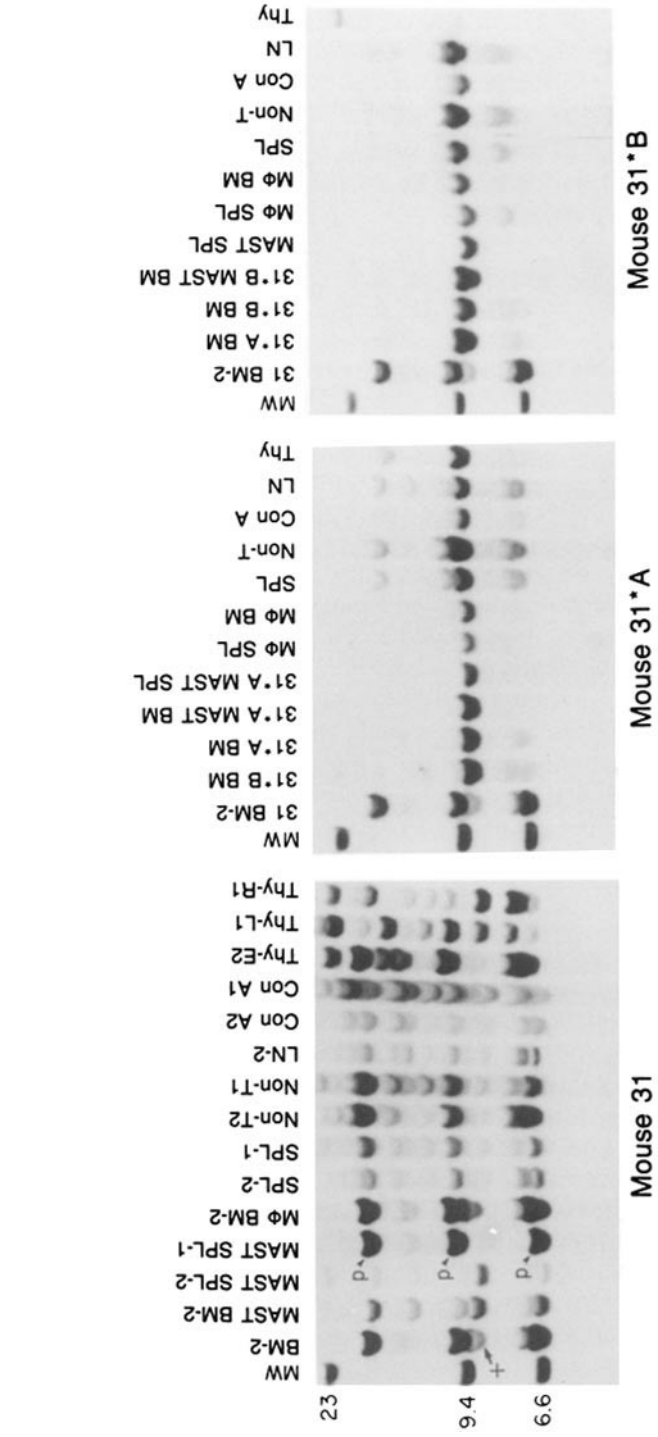

FIGURE 1. Lineage analysis of mouse 31 and its secondary recipient. Mouse 31 was grafted with embryonic thymuses, lethally irradiated, and reconstituted with retrovirally marked bone marrow cells. 2 mo after reconstitution part of the spleen and part of each of the grafted thymuses were removed for clonal analysis. 7 mo after reconstitution an injection of 5-FU was administered. 3 mo after the 5-FU treatment (10 mo after reconstitution) the mouse was killed and various tissues and subpopulations were prepared as described in the Materials and Methods. The abbreviations of tissues and subpopulations are as follows: BM, bone marrow; MAST, mast cells; MΦ, macrophages; SPL, spleen; Non-T, splenocytes depleted of T-cells; Con A, splenic-derived Con A blasts; Thy-L, Thy-R, Thy-E, thymic grafts under the left and right kidney capsule and the endogenous thymus, respectively; LN, lymph node; MW, molecular weight markers. The suffix refers to the time of harvesting the tissue; i.e., (1) first time point (2 mo), (2) second time point (10 mo). Bone marrow cells were harvested at the second time point and used to reconstitute two secondary recipients, 31*A and 31*B (5×10^6 cells per recipient). These secondary recipients were analyzed 7 mo later. DNA (10 μg) was digested with Hind III and analyzed by normal blotting procedures with a probe specific for the *neo* gene of the provirus. (P) Those clones that persisted as major clones in most tissues at both time points. (+) The clone that repopulated both of the secondary recipients. At the time mice were killed, 25%, 56%, and 34% of the in vitro colony forming cells from mice 31, 31*A, and 31*B, respectively, were resistant to 1 mg/ml (active substance) G418.

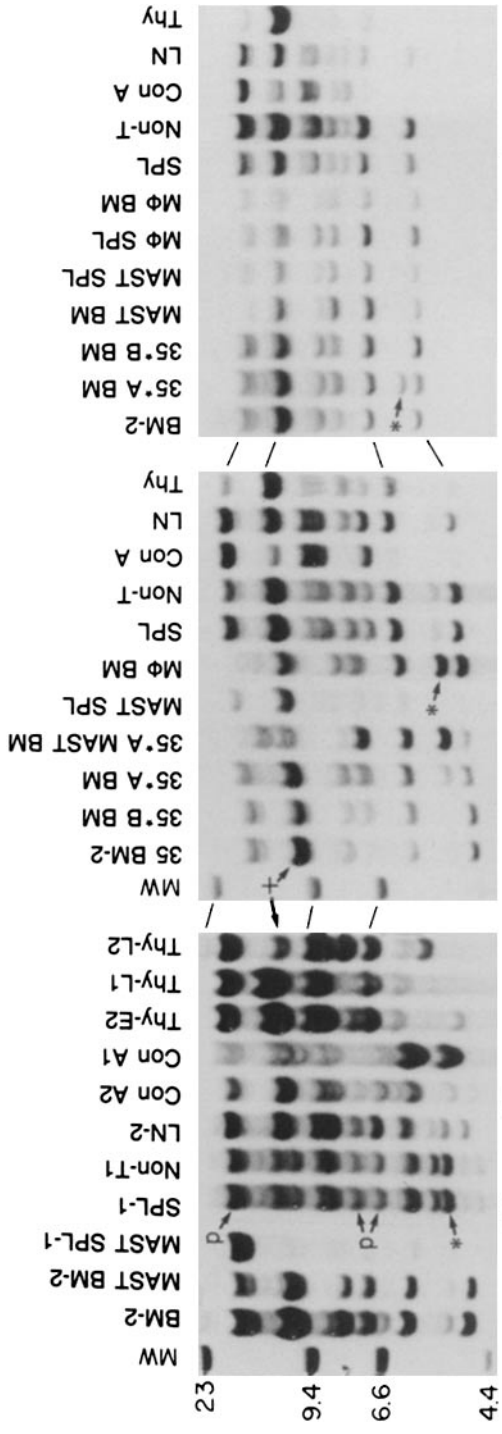

FIGURE 2. Lineage analysis of mouse 35 and its secondary recipient. Mouse 35 and its secondary recipients were treated and analyzed as described in the legend to Fig. 1. (P) Those clones that persisted as major clones in most tissues at both time points. (+) The major clone that repopulated most of the lineages of both of the secondary recipients. (*) A minor clone found in the bone marrow of the primary recipient and in the tissues of only one of the secondary recipients. At the time mice were killed 13%, 24%, and 16% of the in vitro colony-forming cells from mice 35, 35*A, and 35*B, respectively, were resistant to 1 mg/ml (active substance) G418.

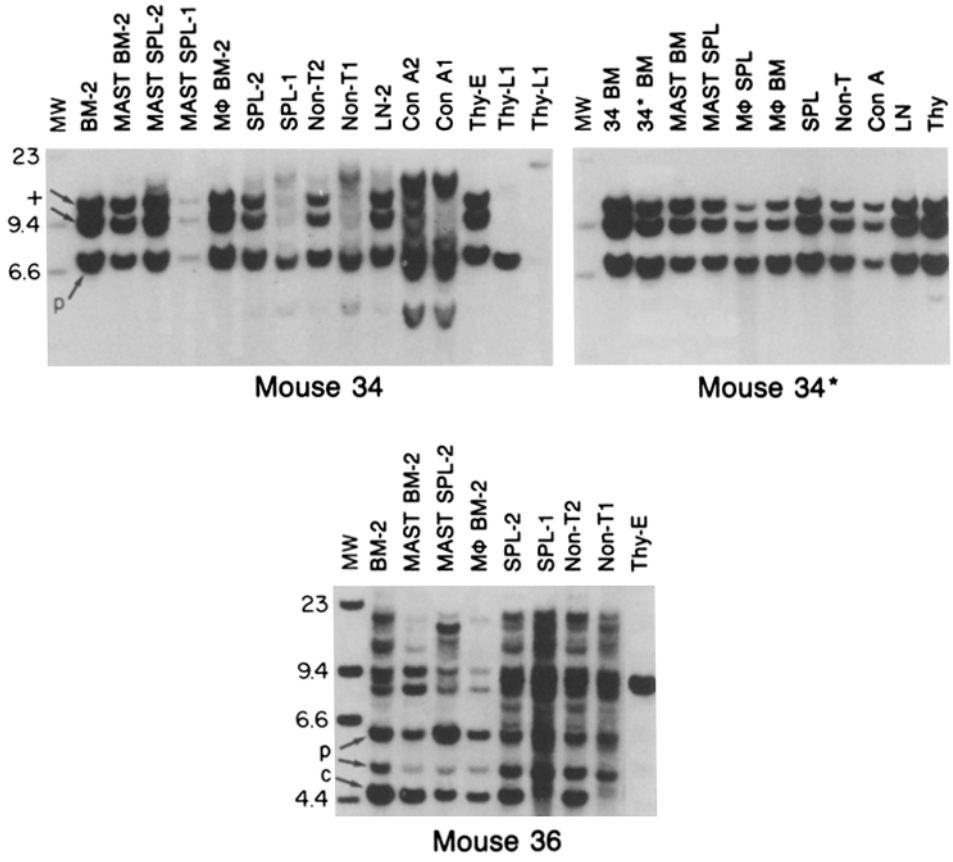

FIGURE 3. Lineage analysis of mouse 34 and its secondary recipient and mouse 36. These animals were treated and analyzed as described in the legend to Fig. 1 except that mice 34 and 36 did not receive an injection of 5-FU. For mouse 34, (P) A clone that was identified as a major clone in all tissues to the 10-mo time point and then persisted to the 17-mo time point in the secondary animal. (+) Marks 2 clones which showed changes in the primary recipient between the 2- and 10-mo time points, and then persisted as predominant clones in the secondary recipient. In mouse 36, (P) clones could be detected at both the 2- and 10-mo time points, and (C) a new clone appeared between these two time points. At the time mice were killed >60%, 82%, and 24% of the in vitro colony forming cells from mice 34, 34*, and 36, respectively, were resistant to 1 mg/ml (active substance) G418.

stable clones, we also identified clones that changed between the 2- and 10-mo time points. The most common changes that we observed was an increase in the relative sizes of existing clones as observed by an increase in the intensity of bands corresponding to these clones. An example of this type of change can be seen in the total spleen (SPL) and the non-T portion of the spleen in mouse 34 (Fig. 3). At the time of the first analysis these tissues contained one predominant band (marked with a "P") plus a number of faint bands. By 10 mo after reconstitution, two of these faint bands had increased significantly in intensity (marked with a "+"), a change that indicates that the corresponding clones have increased in size during this time period. A more extreme example of this type of change can be found in mouse 31 (Fig. 1).

The clone represented by the band at 9 kb (marked with "+") is present in the bone marrow, as well as the mast cells and the macrophages derived from it, and in the splenic mast cells at the second time point. It can also be detected in the Bam HI digests of the whole spleen and in the non-T portion of the spleen at this time (Fig. 4). After a longer exposure of the autoradiograph this clone can be detected as an extremely faint band in the DNA from the grafted thymus indicating that it had begun to seed the thymus at the 2-mo time point, although this was not able to be confirmed by a second enzyme digest (data not shown). It is not, however, detectable in any of the other tissues or cell populations at this time.

A final example of a clonal change with time can be seen in mouse 36. The clone represented by the 4-kb band in mouse 36 (marked with "c") is not present in the spleen, or in the non-T portion of the spleen at 2 mo after reconstitution, but is present in all tissues at 10 mo (Fig. 3). Unfortunately, we were unable to recover any tissue from the grafted thymuses in this animal and thus were unable to sample this tissue at an early time point. The N2 virus packaged with the $\psi 2$ cell line is known to produce helper virus at low levels (20). In our previous studies we have observed that this results in varying levels of viremia (7). This potential source of viral spread is a difficulty only in interpreting newly arising clones. The possibility can not be ruled out that such clones may be a result of newly infected cells. In this study clone "c" in mouse 36 is the only clone discussed that is subject to this possible interpretation.

Taken together, the findings from the initial part of the analysis of these animals confirm our previous observations, namely that the hematopoietic system of irradiated reconstituted mice is made up of clones that show no changes over extended periods of time as well as those that undergo some temporal changes.

Analysis of Secondary Recipients. In general, the clones that were found in the hematopoietic tissues of the secondary recipients could be identified in the primary recipients. The most striking example of this persistence of clones is found in the

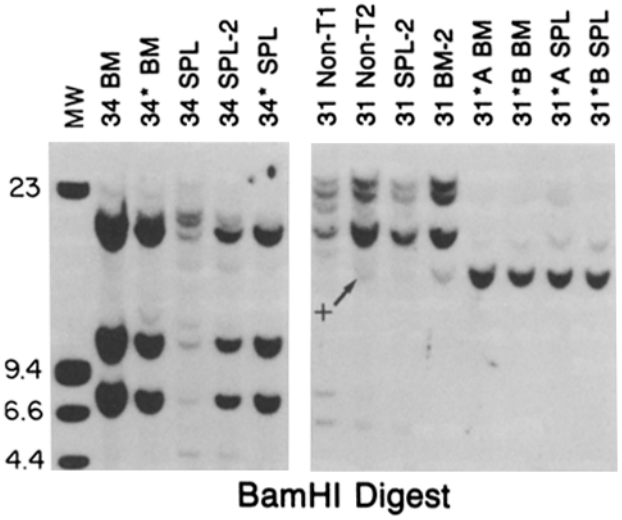

FIGURE 4. Lineage analysis of mouse 34, 31 and their secondary recipients. DNA from selected tissues and subpopulations from the indicated mice was digested with Bam HI and analyzed as described in the legend to Fig. 1. (+) A new stem cell-derived clone that appears at the second time point and repopulates both secondary recipients.

tissues and hematopoietic cell populations of mouse 34* (Fig. 3). Because most if not all of this animals' hematopoietic cells are marked with the N2 virus (see Fig. 3, legend), it seems that its entire hematopoietic system (myeloid and lymphoid) consisted of the same three predominant clones that were present in the hematopoietic tissues of the primary recipient. One of these clones (marked "P") could be easily detected in all of the tissues of the primary animal at 2 mo and all three were present in the mast cells at this early time point. This finding demonstrates that the stem cells that generated these three clones were functioning as early as 2 mo after reconstitution and continued to function throughout the life of the primary recipient and for the duration of the experiment in the secondary host. The minimum life span of these stem cells was 15 mo.

The clonal make-up of the tissues and cell populations from mice 31*A and 31*B was quite different from those of the primary recipient, mouse 31 (Fig. 1). Whereas the hematopoietic tissues of mouse 31 contained many clones, those of the two secondary recipients contained one predominant clone. Moreover the same clone appears to have reconstituted both of these animals indicating that the stem cell which gave rise to it was able to generate a minimum of two new stem cells, each capable of long-term reconstitution. As described above, this particular clone could be identified in the myeloid tissues and cell populations at the second time point and as an extremely small component of the grafted thymus tissue at the time of the first analysis of mouse 31. The same basic pattern can be identified if the DNA is digested with a second enzyme, i.e., that the tissues from both secondary recipients contain the same predominant clone that can be detected as a relatively small clone in the tissues of the primary recipient at the second time point (Fig. 4).

In contrast to these animals, the clonal make-up of the secondary recipients 35*A and 35*B is remarkably similar to that of the primary recipient (Fig. 2). By comparing the bone marrow of these two animals to that of the primary recipient it is possible to identify at least seven common integration sites, indicating that these clones that were present in the primary reconstituted mouse were able to repopulate the hematopoietic tissues of both secondary recipients. Many of these clones could be found in most tissues and cell populations, indicating that they originated from a multipotential stem cell. The presence of identical clones in both secondary recipients would again indicate that the stem cells in the primary recipient were able to generate a minimum of two new stem cells during the course of reconstitution.

Interestingly, one clone (marked "*"), present as a minor clone in the BM of mouse 35, was found only in a number of the tissues from mouse 35*A, but not in any of the tissues or cell populations from mouse 35*B. The thymuses of both secondary recipients were colonized by the same predominant clone. This clone was already detectable in the grafted thymus of the primary recipient at the first time point and the appears to have expanded to become predominant in most tissues by 10 mo and finally persisted to colonize virtually all the tissues of both secondary recipients. This would indicate that the stem cell that gave rise to this clone also had the capacity to function over a 15-mo period.

Discussion

The data presented in this report document the existence of a class of primitive multipotential stem cells that can function for a significant portion of the lifetime

of a mouse (15 mo). These stem cells had the capacity to reconstitute all lineages of the hematopoietic system of an irradiated mouse for a minimum of 8 mo and then continue to function for a further 7 mo when transplanted to a secondary recipient. These findings do not support a strict clonal succession model of hematopoiesis that proposes that the hematopoietic system is maintained by a succession of clones derived from stem cells with a relatively short life span (6, 8–11). In contrast, our data would indicate that at least part of the hematopoietic system of irradiated, reconstituted mice is maintained by long-lived stem cells.

A number of other studies have also provided evidence in support of the existence of such long-lived stem cells. Boggs et al. (12, 13) found that limiting numbers of bone marrow cells could reconstitute W/W^v recipient mice for more than 2 yr and then continue to function in secondary and in some instances tertiary recipients. From a statistical analysis, it is clear that very few stem cells were functioning over this period of time. However, due to the fact that the cells were not uniquely marked, it was impossible to unequivocally demonstrate that an individual stem cell was actually functioning during this time. In a more recent study, Harrison et al. (14) analyzed the variation in the type of hemoglobulin found in reticulocytes of mice reconstituted with two populations of BM cells that could be distinguished from one another on the basis of the electrophoretic mobility of their hemoglobin. From this analysis, they found little change with time (up to 295 d after reconstitution) in the proportion of each type of hemoglobin present in the reticulocyte population, even in mice reconstituted with near limiting numbers of BM cells. This would suggest that the same stem cells were producing progeny over this extended period of time. The existence of long-lived stem cells does not rule out a contribution by short-lived precursors to the hematopoietic system of a reconstituted mouse. In fact, immediately after reconstitution it is likely that short-lived precursors generate the majority of the blood cells, and it is only with time that one can identify the progeny of the primitive stem cells. This then raises the important question of when, following reconstitution, is it possible to detect the progeny of these long-lived stem cells. In a number of our recipients we could identify clones at the first time point (2 mo) that persisted to the time of the second analysis and in some instances contributed to the reconstitution of the secondary recipients. This would suggest that by 2 mo after reconstitution the hematopoietic tissues of an irradiated mouse are reconstituted, to some extent, by progeny of primitive stem cells. However, in some animals the progeny were not easily identifiable in all lineages at this time point (e.g., mouse 34, SPL and non-T). In our previous study (7) we also found that some clones could be detected earlier in some lineages than in others. We have interpreted this type of change as being related to the turn-over rate of the lineage or subpopulation, and we suggest that it reflects the normal establishment and expansion of a clone derived from a primitive multipotential stem cell. A more complete analysis at different times after reconstitution would be required to determine when progeny from the majority of the stem cells can be identified in the different lineages. Our findings do, however, provide some time frame as to when the progeny of primitive stem cells can be detected, and indicate that one should analyze mice beyond the 2-mo time point when measuring stem cell function.

Contributions from short-lived precursor cells could play a more significant role in situations in which the bone marrow is manipulated extensively before reconsti-

tution, since such manipulations could be harmful to the stem cells. With regard to the protocol we have used, it is likely that many stem cells do not survive the co-culture and the preselection steps, as it is not known which culture conditions best support the survival of such cells. To ensure that some stem cells survive these procedures we routinely use large numbers of bone marrow cells as a starting population, and in our more recent studies we have infected the cells with virus-containing supernatant rather than by co-culture (21), since it is possible that the reconstituting cells are adherent and therefore could be lost by sticking to the virus-producing cells. If one uses relatively small numbers of cells in the starting population it is conceivable that, in some instances, the most primitive stem cells are not transplanted and limited reconstitution is achieved by intermediate stage precursors. Under these conditions one would expect some clonal fluctuation within the hematopoietic system of these animals followed by a gradual take-over by host-derived cells, or death of the animal.

A number of reports that demonstrate clonal fluctuation within the hematopoietic system of the mouse can also be interpreted as supporting the concept of long-lived stem cells. Micklem and Loutit (10) followed the fate of clones marked with radiation-induced chromosomal translocations through several transplanted recipients over a period of 2 yr. Although some fluctuation in the size of these clones was found after transplantation, two of the three clones analyzed did persist throughout the experiment. Mintz et al. (8) analyzed the erythroid population of mice that were reconstituted during fetal development with limiting numbers of a mixture of fetal liver cells. From the data reported, four of eight mice appeared to be stably reconstituted for up to 1 yr with cells from only one of the donors, an observation which supports the existence of long-lived reconstituting cells. The most notable fluctuations were found in animals that contained significant numbers of host cells indicating that perhaps there was an incomplete engraftment of primitive stem cells. In a more recent study (11), Capel et al. followed the clonal make-up of the hematopoietic system of W/W^v mice reconstituted with retrovirus-transduced stem cells, and they found, as we have, clones that persisted as well as clones that underwent some temporal changes. A number of these changes could represent the expansion of clones from primitive stem cells during the course of the analysis.

Finally, Lemischka et al. (6) followed the fate of retrovirally marked stem cells when BM cells were passaged from primary reconstituted animals to secondary recipients. In one instance, a number of new clones were detected in the tissues of the secondary animals. However, the predominant clone from the primary recipient was also present in this animal, suggesting that this clone was derived from a relatively long-lived stem cell. In a second transplantation, they reconstituted five secondary recipients from one primary animal and found no new clones. They also analyzed, at different time points after reconstitution, the clonal make-up of cells from the peripheral blood of primary reconstituted animals. Although some changes were noted, the basic pattern remained remarkably stable, suggesting that the mice were reconstituted with long-lived stem cells.

It is clear from the analysis of the secondary recipients of mice 34 and 35 in this report that many of the stem cells that functioned up to 10 mo in the primary animals were able to continue functioning for a further 7 mo in the new host. This finding suggests that each of these stem cells exhibited some capacity to self-renew. On the

other hand, many of the clones found in mouse 31 were difficult to detect or were present at a significantly reduced size in the secondary hosts, indicating that the stem cells that they were derived from might not have clonally expanded to the same extent, or may not have survived the transplantation procedure. The predominant clone in the secondary animals appears to have expanded in the primary recipient sometime between the second and tenth month. Whether or not the expansion of this clone is related to the 5-FU treatment is at present not clear. The observation that both pairs of secondary recipients, i.e., mice 35*A/35*B and mice 31*A/31*B, were repopulated by the same clones suggests that each of the stem cells that they originated from were able to generate a minimum of two new stem cells in the course of reconstituting the primary recipients. The possibility exists that the cells that reconstituted these secondary recipients are not multipotential stem cells, but rather committed precursors derived from the original infected stem cell. However, in light of the fact that the existence of committed precursor cells with long-term reconstituting capacity has not been unequivocally demonstrated, as well as the fact that the clonal make-up of the different lineages is so similar, it is likely that the reconstituting cells of the secondary recipients are indeed multipotential stem cells. This finding is the first indication that this population of long-term reconstituting stem cells can actually expand during regeneration of the hematopoietic system.

Summary

The findings reported in this study highlight several important features of the development of hematopoietic stem cells after transplantation into irradiated recipients. First, they demonstrate the existence of a class of primitive multipotential stem cells that can function for a significant portion of the lifetime of a mouse (15 mo). In addition, they clearly show that these primitive stem cells can be infected with recombinant retroviruses and thus would be appropriate targets for gene therapy in somatic tissues. Second, our data indicate that the progeny of some, but not all, of the primitive stem cells have fully expanded into the various hematopoietic lineages by 2 mo after reconstitution. Finally, our analysis of the secondary recipients provides strong evidence suggesting that the primitive stem cell population can actually clonally expand. Our current experiments are aimed at determining the extent to which this expansion can occur and whether or not this expansion can be influenced by exogenous factors.

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