Fractionation of Mouse Bone Marrow by Adherence Separates Primitive Hematopoietic Stem Cells From In Vitro Colony-Forming Cells and Spleen Colony-Forming Cells

By Friedemann Kiefer, Erwin F. Wagner, and Gordon Keller

Fractionation of mouse bone marrow by adherence to tissue culture plastic was used to characterize the adhesive properties of hematopoietic stem (HS) cells capable of long-term reconstitution. The adherent fraction that represents approximately 13% of the total marrow population was virtually devoid of in vitro colony-forming cells and spleen colony-forming cells but did contain approximately 30% of the total HS cells recovered from the procedure. These cells could be detected by both the competitive repopulation assay and by repopulation of W/W' recipients. In approximately 60% of the recipients from the competitive repopulation experiments, the contribution of the adherent marrow cells was relatively low early (8 to 10 weeks) after transplantation. With time, however, the hematopoietic contribution from these cells increased, reaching a stable level 20 to 30 weeks posttransplantation. In the remaining recipients (40%), the contribution from adherent cells was already significant within 8 to 10 weeks of transplantation and did not change dramatically throughout the course of the experiment. Adherent bone marrow containing significant numbers of HS cells was unable to protect mice from radiation death, indicating that these early cells in the absence of later-stage progenitors are unable to provide this function.

© 1991 by The American Society of Hematology.
CFC or CFU-S adhere, indicating that this method provides a simple means of separating primitive stem cells from the more mature precursor populations.

MATERIALS AND METHODS

Mice. C57BL/6 mice, which carry the Gpi-1<sup>+</sup> allele, and congenic C57BL/6 CAST-Gpi-1<sup>+</sup> mice, which carry the electrophoretically distinguishable Gpi-1<sup>-</sup> allele, were used for the competitive repopulation analysis. WBB6F<sub>1</sub>-W/W<sup>+</sup> anemic mice were used as recipients for the limiting dilution analysis. All animals were bred and housed at the animal care facility at the Institute of Molecular Pathology (IMP) in Vienna.

Adherence procedure. BM cells were suspended in prewarmed (37°C) Iscove's modified Dulbecco's medium (IMDM) supplemented with 5% fetal calf serum (FCS) and seeded at a density of 2 x 10<sup>7</sup> cells in 10 mL in 100 x 15 mm tissue culture dishes (Nunc, Roskilde, Denmark). Following 2 hours of incubation at 37°C in an environment of 5% CO<sub>2</sub> in air, the nonadherent cells were removed after gentle agitation of the dish. The adherent population was washed four times, each time with 10 mL of warm IMDM. The cells removed by washing were added to the nonadherent population. Those cells that remained adherent to the dish after this washing procedure were considered to be the adherent population and were recovered by a short trypsinization followed by gentle scraping with a cell scraper.

Competitive repopulation. We have adapted the competitive repopulation assay used by Harrison<sup>2</sup> quantitated long-term repopulating stem cells (HS cells) in both the adherent and nonadherent marrow fractions. BM from either B<sub>6</sub>-Gpi-1<sup>+</sup> or B<sub>6</sub>-Gpi-1<sup>-</sup> mice was fractionated by adherence. Cells from these fractions were injected intravenously with a standard amount of congenic competitor marrow (carrying the other Gpi-1 allele) into irradiated (9.5 Gy, 60Co, at 0.95 Gy/min) recipient mice. In each experiment, the competitor marrow and the recipient animal were of the same Gpi-1 type. The donor cells were usually obtained from male mice and were injected into female recipients. Typically, the equivalent of ½ of a femur representing between 1.0 x 10<sup>7</sup> and 1.5 x 10<sup>7</sup> competitor cells was injected together with varying numbers of cells from either the adherent or nonadherent fraction. At different times after transplantation, peripheral blood (PB) of the recipient was analyzed for the relative proportions of experimental and competitor marrow. The content of long-term repopulating stem cells (HS cells) in each fraction was calculated from the ratio of Gpi-1A to Gpi-1B (% Gpi-1A/Gpi-1B) isoenzyme activity found in the PB beyond 30 weeks of transplantation. For the purpose of this calculation, it was assumed that 1% of competitor cells contained approximately 30 stem cells, an estimate obtained from limiting dilution repopulation of W/W<sup>+</sup> recipients.<sup>5</sup>

Clonal assays. For the CFU-S assay, irradiated recipients were injected with varying numbers of adherent, nonadherent, or unfractonated marrow cells. Twelve days after transplantation, the animals were killed and the number of colonies in the spleen counted under a dissection microscope. In vitro CFC were assayed in methyl cellulose cultures under the condition described previously.<sup>22</sup>

Glucose phosphate isomerase (GPI) analysis. Cells from PB or from the various tissues were lysed in hypotonic lysis solution (1 mg/mL EDTA) by three cycles of freezing and thawing. Clear lysates were prepared by 10 minutes of centrifugation at 14,000 rpm. Separation of the isoenzymes by electrophoresis on cellulose acetate membranes (Helena no. 3023; Helena Laboratories, Beaumont, TX) and subsequent visualization of the enzymatic activity has been described elsewhere.<sup>1</sup> Gels were scanned using a Bio-Rad Model 620 video densitometer (Bio-Rad, Richmond, CA).

RESULTS

The adherent fraction of mouse BM is virtually devoid of CFC and CFU-S. The distribution of nucleated cells, CFC, and CFU-S in the adherent and nonadherent marrow fractions is outlined in Table 1 and Fig 1. Approximately 75% of the starting marrow population was recovered following the adherence procedure described in Materials and Methods. Of the cells recovered, between 10% and 15% were found in the adherent fraction. Analysis of the precursor content of the adherent population indicated that the frequency of CFC and CFU-S was dramatically reduced compared with the nonadherent population or to unfractonated marrow (Table 1). This finding was true for the CFC committed to a single hematopoietic lineage as well as for the CFC able to generate multilinage colonies. The frequency of these precursor populations in the nonadherent fraction was not significantly different from that of unfractonated marrow, indicating that a large majority (> 95%) of these cells are nonadherent (Fig 1).

The adherent marrow fraction contains HS cells capable of long-term repopulation. Both the adherent and nonadherent fraction were analyzed for the presence of HS cells capable of long-term repopulation. To be able to quantitate this population within each fraction, the analysis was performed by competitive repopulation.<sup>6</sup> In four separate experiments, long-term repopulating cells were detected in the adherent fraction. These cells had the characteristics of HS cells as they were able to generate mature hematopoietic cells for the duration of each of the experiments, which lasted between 30 and 55 weeks.

In many recipients, the full capacity of the adherent population to form mature blood cells was not readily detectable early after transplantation, which is most likely due to the absence of later-stage precursor cells in this fraction. With time the proportion of PB cells derived from the adherent fraction increased relative to those originating from the competitor marrow. A steady state was reached between 20 and 30 weeks after transplantation. In contrast, the hematopoietic contribution from the nonadherent population tended to decrease over the same period of time, although these changes were not as dramatic as those observed in recipients with adherent marrow.

The kinetics of repopulation from one experiment are depicted in Fig 2. Seven weeks after transplantation, cells from the adherent fraction contributed very little (<10%) to the PB of three independent recipients (Fig 2A; mice

<table>
<thead>
<tr>
<th>Adherent Fraction</th>
<th>Nonadherent Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU/10&lt;sup&gt;6&lt;/sup&gt; cells*</td>
<td>371 ± 209</td>
</tr>
<tr>
<td>CFU-S/10&lt;sup&gt;6&lt;/sup&gt; cells†</td>
<td>30</td>
</tr>
</tbody>
</table>

The frequencies are given per 10<sup>6</sup> nucleated cells recovered following the adherence procedure. Abbreviations: CFC, CFU in a standard methylcellulose assay; CFU-S, day 12 CFU-S.

*Frequency of CFC is the mean ± SD of four experiments.
†Frequency of CFU-S is the mean of two experiments.
experiment. In four independent experiments, 17 of 30 reconstituted animals showed an increase (on average 36% ± 19%) in the blood-forming capacity of the adherent BM cells. Of those recipients that received nonadherent BM cells, 13 of 22 showed a decreasing contribution (on average 15% ± 10%) to the PB over time. The remaining recipients of both adherent and nonadherent marrow showed a relatively stable pattern of reconstitution throughout the analysis. The contribution from the adherent population ranged from 30% to 60% in most of these recipients. These findings clearly show that the adherent population does contain stem cells capable of long-term reconstitution. In addition, they show that the contribution to hematopoiesis of these cells is not always readily detectable immediately after transplantation.

The stem cell content of both fractions was determined by comparing the reconstituting potential of each with the unmanipulated competitor marrow. This analysis was performed relatively late after reconstitution, when the contribution from the experimental populations had stabilized. In three separate experiments, it was found that 30% of the total stem cell population capable of long-term reconstitution was present in the adherent fraction (Fig 1), representing an enrichment of approximately twofold over unfractionated marrow.

All of the analyses outlined above are based on the hematopoietic contribution of the experimental marrow to the PB. To ensure that the composition of the PB accurately reflects the clonal makeup of the entire hematopoietic system, BM, spleen, and thymus from 12 recipients were analyzed for the proportion of cells derived from the experimental populations. The data from four of these animals are shown in Fig 3. The relative proportion of each donor population in the spleen and BM is similar to that found in the PB. In several recipients, the contribution of the fractionated marrow to the thymus was somewhat lower than the contribution to the other tissues. This occurred in animals in which the thymus was significantly atrophied at the time of analysis and thus could reflect a major contribution of host tissue in the sample analyzed.

Moreover, in the BM of three long-term reconstituted recipients (including animals 22B and 22R) the presence of different myeloid progenitors derived from the experimental marrow fraction was shown by GPI isoenzyme analysis of individual hematopoietic colonies picked from methyl cellulose cultures. Multilineage, macrophage, mast-cell, and granulocyte-macrophage colonies derived from the adherent fraction were detected (data not shown).

Determination of the HS cell frequency in the adherent BM fraction by limiting dilution into W/W mice. As an independent means of analyzing the HS cell content of the adherent population, BM cells from the adherent fraction were transplanted into mutant W/W" mice. Because wild-type hematopoietic cells have a growth advantage over those from the mutant mice, 23 irradiation before transplantation is not required and, as a consequence, relatively small numbers of HS cells can be analyzed through limiting dilution analysis. A total of 83 W/W" recipients was reconstituted with varying numbers (ranging from 1 × 10^6

---

**Fig 1.** Distribution of nucleated cells (NBC), CFC, CFU-S, and HS cells between the adherent and the nonadherent fraction of mouse BM. Results are given as percentage of the total recovery of each cell type after 2 hours of adherence. Data points for NC and CFC are the mean ± SD from four experiments. Data points given for CFU-S and HS cells are the mean ± SD from two and three experiments, respectively.

22R, 22B, and 22L). By week 16 after reconstitution, the contribution from the adherent fraction had increased significantly (53%) in one of the animals (22R), only marginally (20%) in another (22B), and remained unchanged in the third (22L). By week 23, the adherent cell contribution had increased to a stable level in all of the recipients. Although the degree of change varied significantly in these recipients (20%, 50%, and 80%), the kinetics of change was similar. In three recipients transplanted with the nonadherent marrow fraction (Fig 2B), the contribution of these cells to hematopoiesis tended to decrease (90% to 65%; 70% to 60%) with time. The most significant decrease was observed within the first 16 weeks after reconstitution, after which time a relatively stable pattern of reconstitution persisted for the duration of the experiment.
approximately 1 in 25,000 adherent marrow cells (Fig 4). A similar frequency was calculated using the $\chi^2$ method of analysis described by Porter and Berry.\(^{24}\) Tissues from individual recipients were also analyzed for contribution from the adherent population. As in the previous experiments, those mice that contained cells derived from adherent marrow in the PB also contained these cells in the different hematopoietic tissues (data not shown).

Adherent marrow cells are inefficient at protecting mice from radiation death. The late onset of adherent marrow-derived hematopoiesis after transplantation suggested that these cells might be inefficient at protecting mice from radiation death. To determine the radioprotective potential of the adherent marrow subpopulation, varying numbers of these cells (between $1 \times 10^5$ and $5 \times 10^5$) were transplanted into irradiated recipients in the absence of competitor marrow. Aliquots of the same adherent fractions were also transplanted together with competitor marrow to show the presence of HS cells. In two separate experiments, 13 of 14 recipients that exclusively received cells from the adherent population died between 1 and 2 weeks after irradiation (Table 2) and only one mouse survived. This result was true for animals that received as many as 32 long-term repopulating HS cells. The same number of adherent cells was able to contribute significantly to the hematopoietic system of the

Fig 3. GPI isoenzyme composition of different hematopoietic tissues from four mice. At 44 weeks after BM reconstitution, samples of PB, BM, spleen (S), and thymus (T) were taken. The spleen and thymus explants were rinsed extensively in phosphate-buffered saline. After passage through a metal mesh, contaminating erythrocytes were removed from these cell suspensions by lysis in a hypotonic buffer. Cleared lysates were prepared from all samples and GPI-1A isoenzyme activity was determined by serum electrophoresis followed by enzymatic visualization of the different GPI isoenzymes.

Fig 4. Determination of the frequency of HS cells in the adherent BM fraction by limiting dilution into W/W$^+$ mice. (A) Increasing numbers of adherent marrow cells ($1 \times 10^5$ to $1 \times 10^6$) of the Gpi-T$^+$ isoenzyme type were injected into groups of W/W$^+$ mice. At 42 weeks after the injection, the percentage of graft-derived PB cells was determined for each animal by analysis of the GPI isoenzyme activities. Each bar represents the GPI-1A isoenzyme activity in the PB of an individual animal. (B) The percentage of animals without graft-derived PB cells within a group is plotted on a logarithmic scale versus the number of cells grafted. The data points were connected by linear regression (linear correlation = 0.986). According to Poisson's statistics, the frequency of HS cells in the adherent fraction is the abscissa value corresponding to a hypothetical group of 37% animals without graft-derived blood cells.

2580
KIEFER, WAGNER, AND KELLER

to $1 \times 10^6$) of adherent BM cells. Consistent with the competitive repopulation assay, stem cells capable of repopulating the hematopoietic system of the W/W$^+$ recipients were found in the adherent marrow fraction. Using Poisson statistics, the frequency of these cells was calculated to be

\[ 25R \]

\[ 2580 \]

\[ 2 \sim 10'\]
were detected by a number of different methods, including the generation of CFU-S in long-term BM cultures, the colonies of blast cells in methyl cellulose cultures or by term BM cultures. Primitive mouse hematopoietic cells their ability to initiate and sustain hematopoiesis in long-term BM cultures provides a relative simple method for the separation of those studies performed with human cells, primitive hematopoietic systems of both human and mouse, cells are adherent than those representing the later In hematopoietic system of both human and mouse, cells are capable of long-term repopulation can be separated from CFU-S, exploiting their differential affinity to the fluorescent dye rhodamine-123. Jones et al showed that repopulating HS cells could be separated from CFC and CFU-S on the basis of size and density using counterflow elutriation. They found that the fraction that contained the most CFC and CFU-S provided protection from radiation death but was unable to support long-term repopulation. In contrast, the fraction that contained repopulating cells but relatively few CFC and CFU-S was unable to supply significant numbers of mature hematopoietic cells early after transplantation and was thus incapable of protecting mice from radiation death.

**DISCUSSION**

In this report we show that a subpopulation of HS cells capable of long-term repopulation can be separated from most CFC and CFU-S by adherence to tissue culture plastic. With the adherence protocol we have used, approximately 30% of the total HS cell population is present in the adherent fraction as determined by competitive repopulation. This result represents an enrichment of about twofold over unfractionated marrow, given that the adherent population consists of approximately 13% of total BM. The frequency of HS cells in the adherent fraction, as determined by limiting dilution repopulation of anemic W/W<sup>−</sup> mice, was calculated to be approximately 1 in 25,000 cells, a number that is not significantly different from the frequency of HS cells in unfractionated marrow (1 in 30,000). Thus, by two independent quantitative assays, we have shown the existence of substantial numbers of long-term repopulating HS cells in a marrow population that is significantly depleted of the more advanced CFC and CFU-S populations. Adherence to tissue culture plastic, therefore, provides a relative simple method for the separation of those early cells from the more advanced hematopoietic precursor populations.

A number of other studies have suggested that in the hematopoietic system of both human and mouse, cells representing the early stages of development are more adherent than those representing the later stages. In studies performed with human cells, primitive hematopoietic cells were measured by their ability to generate colonies of blast cells in methyl cellulose cultures or by their ability to initiate and sustain hematopoiesis in long-term BM cultures. Primitive mouse hematopoietic cells were detected by a number of different methods, including the generation of CFU-S in long-term BM cultures, the ability to repopulate a long-term BM culture, the production of multilineage colonies in secondary replated methyl cellulose cultures, and the ability to repopulate the hematopoietic system of irradiated recipients for short periods of time. In only one study were the cells transplanted into recipient animals for extended periods of time and in this instance the adherent marrow fraction was found to be more effective than unfractioned marrow in reconstituting the spleen and marrow cellularity of these recipients.

None of these approaches, however, provided a quantitative estimate of the HS cell content of the adherent population.

Recently, a number of other studies have defined characteristics of primitive hematopoietic cells that can be used to physically separate them from later-stage cells. Ploemacher and Brons found that pre-CFU-S, a population able to generate CFU-S in a short-term transplantation assay, can be separated from CFU-S, exploiting their differential affinity to the fluorescent dye rhodamine-123. Jones et al showed that repopulating HS cells could be separated from CFC and CFU-S on the basis of size and density using counterflow elutriation. They found that the fraction that contained the most CFC and CFU-S provided protection from radiation death but was unable to support long-term repopulation. In contrast, the fraction that contained repopulating cells but relatively few CFC and CFU-S was unable to supply significant numbers of mature hematopoietic cells early after transplantation and was thus incapable of protecting mice from radiation death.

Similarly, we have found that populations of adherent marrow that contain significant numbers of long-term repopulating HS cells but few CFC and CFU-S are not able to rapidly generate sufficient numbers of mature hematopoietic cells to protect mice from radiation death. Taken together, these studies clearly show that neither the CFU-S assay nor the radiation protection assay measure the most primitive stem cell population. In addition, they also show that the only reliable assays for these cells are competitive repopulation and reconstitution of W/W<sup>−</sup> recipients because neither is dependent on the rapid generation of mature blood cells from the HS cell population.

The mechanism by which HS cells adhere to plastic is presently unknown. They may adhere directly or, alternatively, they could attach to fibroblasts or other stromal type cells that adhere in turn to the plastic. Regardless of the mechanism of adherence, it is tempting to speculate that it is mediated by a process that plays some significant role in vivo, such as homing of the HS cells to the BM after transplantation. It is also not clear why only a subpopulation of the HS cells adhere. If adherence occurs via another cell type, it is possible that these intermediate cells are limiting. Alternatively, there could be heterogeneity within the HS cell population with respect to adhesive properties. Approaches towards a better understanding of the mechanisms of adherence as well as a search for conditions that could selectively increase the proportion of long-term repopulating HS cells that adhere are currently in progress.

The identification of primitive stem cells within the adherent fraction has important implications for manipula-
tions of BM before transplantation. This is particularly relevant for protocols aiming to infect hematopoietic cells with recombinant retroviruses that are used as experimental mouse models for future gene therapy trials. One of the most common methods of infection is to coculture the hematopoietic cells with fibroblasts that produce a recombinant virus, a procedure that is likely to result in the loss of significant numbers of adherent cells. The alternative method of infection is to culture the hematopoietic cells in a virus-containing supernatant, allowing for recovery of all the adherent cells. In a preliminary study, we have found that threefold to fourfold more stem cells are recovered from a marrow population that has been cultured in a virus-containing supernatant for 18 hours than from a comparable population that was cocultured with virus-producing fibroblasts for the same period of time. However, as little as 10% of the HS cells originally present in infected 5-fluorouracil (5-FU)-pretreated BM were recovered after 18 hours in vitro culture following the supernatant protocol. Thirty percent of the HS cells present in the in vitro cultured population could be recovered when normal BM was infected under the same conditions. These findings indicate that supernatant infection is likely to be the method of choice for retroviral infection of primitive hematopoietic cells. In addition, they highlight the problems involved with manipulating these cells in culture.

In conclusion, we have presented a fast and simple method that allows for the separation of 30% of the long-term repopulating stem cells from the vast majority of the later-stage hematopoietic precursor populations. This adherent BM fraction provides a valuable tool for further characterization of HS cells and can be used as a starting population for the enrichment of HS cells by antibody depletion.

ACKNOWLEDGMENT
We thank Dr Bob Phillips for help with the \( \chi^2 \) analysis and Drs Warren Alexander, Hartmut Beug, Norman Iscove, Martin Nicklin, and Thomas von Rüden for critical comments.

REFERENCES

Fractionation of mouse bone marrow by adherence separates primitive hematopoietic stem cells from in vitro colony-forming cells and spleen colony-forming cells

F Kiefer, EF Wagner and G Keller