Monoclonal derivation of mouse myeloid and lymphoid lineages from totipotent hematopoietic stem cells experimentally engrafted in fetal hosts

(fetal liver/intraplacental microinjection/differentiation/clonal succession/mouse mutant W-series anemias)

BEATRICE MINTZ, KATE ANTHONY, AND SAMUEL LITWIN

Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA 19111

Contributed by Beatrice Mintz, August 24, 1984

ABSTRACT Mutant mouse fetuses with a hematopoietic stem cell defect were injected with a mixture of two normal strains of fetal liver cells to test the possibility of seeding with single stem cells and of deriving all hematopoietic lineages clonally. Recipients were either W^f/W^f , with a mild endogenous defect offering only marginal selective advantage to a normal donor cell, or W/W , with a severe defect. Among 11 W^f/W^f animals with long-term grafts, 8 had only one or the other of the donor strains. Some of these individuals must have been seeded by only a single donor cell ($P = 0.1$); the frequency of this event was at least 20% (90% confidence) and most likely 50% of the cases. Cell-specific strain markers in myeloid and lymphoid lineages reinforced the likelihood that renewal and differentiation had occurred from a totipotent hematopoietic stem cell. In a smaller W/W group, some hosts were seeded by at most two cells ($P = 0.1$), and single-cell seeding could not be ruled out. The experiment allows stem cell pedigrees to be examined during the normal developmental progression. In both groups observed here, some mice displayed a regular and complementary rise and fall in proportions of cells of different genotypes, thereby suggesting clonal succession in a hierarchy of stem cell compartments. This transplant system also offers advantages for future experiments on regulated expression *in vivo* of genes transferred (*in vitro*) into totipotent hematopoietic stem cells.

There is evidence in mammals for the existence of totipotent hematopoietic stem cells (THSC) ancestral to all myeloid and lymphoid blood lineages (1-7). These cells, apparently present in fetal liver and postnatal bone marrow, have not been morphologically identified or isolated and, thus, cannot be individually transplanted. In the present report, we describe a transplantation strategy affording substantial likelihood that a single THSC can become engrafted in an intact (unirradiated) mouse fetus. Self-renewal and differentiation can then be followed throughout life in the clonal progeny of that cell.

W-series mouse mutants have a stem cell defect as well as macrocytic anemia and are able to accommodate and provide a selective advantage to stem cells from normal donors (8). We have devised a means of introducing hematopoietic cells into early fetuses by microinjecting a small inoculum into a placental blood vessel (4). Allogeneic fetal liver cells are accepted with impunity. Using a series of mutant recipients with graded severity of the defect, it became apparent that the least defective ones (e.g., W/W) were relatively infrequently seeded by normal fetal liver cells, whereas severely defective hosts (e.g., W^f/W^f) were much more frequently and rapidly engrafted (5). [With the same size inocu-

FIG. 1. The experimental plan, predicting that conditions in mildly defective (W^f/W^f) mutant hosts would be relatively favorable for prenatal seeding by single hematopoietic stem cells, in tests with a mixed inoculum of two normal (+/+) fetal liver genotypes (solid and open stars).

lum, normal (+/+) fetuses are only rarely and briefly populated by donor cells (unpublished data).] Thus, engraftment in mildly afflicted hosts seemed to entail very few donor cells—possibly often individual ones. We have here tested and verified this possibility by offering to the recipient a “choice” from a mixture of two normal strains of fetal liver cells (Fig. 1).

MATERIALS AND METHODS

Genetic Markers. Recipient fetuses in one group were of the W^f/W^f genotype on an inbred C3H strain background and were from matings between mutant homozygotes, which are mildly anemic and fertile (9). Suspensions of liver cells from 13-day fetuses of the C57BL/6 and BALB/c strains (Institute for Cancer Research sublines) were combined in approximately equal numbers, and $1-2 \times 10^5$ cells were injected into the placental circulation of 11-day fetal hosts as described (4). The proportion of erythrocytes (RBC) specifically from the C57BL/6 donor was determined postnatally from the percentage of the *single* (Hbb^s/Hbb^s) electrophoretic variant of the β -chain of hemoglobin; BALB/c and C3H have the *diffuse* (Hbb^d/Hbb^d) variant. The proportion of RBC specifically from the BALB/c donor was determined from the percentage of glucose phosphate isomerase (GPI;

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: THSC, totipotent hematopoietic stem cell; RBC, erythrocyte; GPI, glucose phosphate isomerase.

D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9) of the *Gpi-1^a/Gpi-1^a* electrophoretic type; C57BL/6 and C3H are *Gpi-1^b/Gpi-1^b*. Some congenic C57BL/6 donors had the *beige* (*bg^f/bg^f*) marker cytologically detectable in neutrophilic granulocytes (10). Strain-specific variants of immunoglobulin allotypes (*Igh-1* locus) were used to distinguish B-lymphocyte strains.

Recipient fetuses in the other group were from matings between *W/+* heterozygotes to obtain the severely anemic *W/W* type that is ordinarily lethal shortly after birth and is distinguishable by coat color from *W/+* and *+/+* littermates. One *W/+* parent was on the C57BL/6 strain background, except for substitution of *Hbb^d/Hbb^d* for *Hbb^s/Hbb^s*; the other was on the WH strain background (*Hbb^d/Hbb^d*; *Gpi-1^b/Gpi-1^b*). With the same mixture of C57BL/6 and BALB/c donor cells as in the first group, the hemoglobin and GPI markers again specifically distinguished RBC derived from the respective donor strains. In one case, an AKR donor was used in place of BALB/c, with the same markers.

Blood Analyses. Blood was collected at intervals from the tail tip or retro-orbital sinus. RBC lysates were electrophoresed to separate hemoglobin variants (11), and the transparentized gels were densitometrically scanned (5). GPI in the lysates was electrophoresed in cellulose acetate (12) and stained as described (13) with the addition of 2% agarose, and the variants were estimated visually. Fixed blood smears were stained (14) to identify *bg^f/bg^f* granulocytes with giant sudanophilic lysosomes. Immunoglobulin allotypes were determined by the Ouchterlony double-diffusion assay.

RESULTS AND DISCUSSION

Of 236 *W^f/W^f* mildly defective recipients, 32 (14%) had donor RBC, usually transiently during the first postnatal week at a level of less than 10%; 11 (5%) of these remained positive for at least 8 weeks (Table 1). The life-span of mouse RBC is 6–7 weeks (15); therefore, a self-renewing population of donor stem cells must have been established in these hosts. Engraftment occurred at a frequency similar to that found (6%) when only one strain of fetal liver cells was administered to *W^f/W^f* mice in the same-size inoculum (5).

Of the engrafted *W^f/W^f* mice, 8 had RBC of only one or the other donor type, and 3 had both RBC strains (Table 1). As in our earlier studies with a single donor strain (5), engraftment could be classified as “complete” when donor RBC persisted at close to 100% or as “limited” in either extent or duration of replacement. Six of the 11 animals had “complete” replacement, including 4 from the C57BL/6 donor, 1 from BALB/c, and 1 from both; 5 had “limited” replacement, including 2 from C57BL/6, 1 from BALB/c, and 2 from both. The time course in 6 of the *W^f/W^f* hosts is shown in Fig. 2.

In our previous experiments, with either fetal liver (5) or adult bone marrow (7) grafts, progressive replacement of donor-type RBC was invariably accompanied by coordinate re-

placement in other myeloid and in lymphoid lineages whenever they were tested. Thus, RBC replacement was a reliable indicator of engraftment at the THSC level under these conditions. In the present study, developmental totipotency of the engrafted cells was again verified in three of the *W^f/W^f* hosts with “complete” replacement (97–100% C57BL/6 donor RBC) by the *bg^f/bg^f* congenic derivative of C57BL/6. The same animal as shown with 100% C57BL/6 RBC at age 55 weeks (Fig. 2A) had the diagnostic *bg^f/bg^f* granulocytes (Fig. 3 *Left*) and the C57BL/6 immunoglobulin allotype (Fig. 3 *Right*) at that age.

In a smaller series, 46 *W/W* severely deficient segregants were obtained from fetuses injected in *W/+* matings. Donor-strain RBC were present at least transiently in 18 (39%) of the *W/W* mice; 9 (20%) of these had donor-derived RBC for >8 weeks, including 6 cases with one donor strain (Fig. 4) and 3 cases with both (Table 1). Unlike the situation in *W^f/W^f*, the “complete” type of replacement was much more prevalent than the “limited” type—a result consistent with the greater severity of the *W/W* host's disease.

Roughly equal numbers of both donor cell strains were injected; hence, if many donor cells were seeded in each recipient, it seems unlikely that any mouse would escape seeding by both donors. However, 8 of 11 *W^f/W^f* mice had only one donor genotype (Table 1). Our statistical analysis (below) shows that the assumption that every animal was seeded with at least two cells was incompatible with the *W^f/W^f* data (significance level = 0.1) and that at least 1 of these 8 individuals must have been seeded by a single donor cell. We estimate that 1.32 cells per mouse (maximum likelihood estimate) were seeded; this corresponds to 48.1% of successfully engrafted *W^f/W^f* mice seeded by only a single cell. A lower one-sided 90% confidence bound on this fraction is 21.3%.

A similar analysis applied to the more limited *W/W* data permit only the weaker conclusion that some of these mice were seeded by not more than two cells, but single-cell seeding cannot be ruled out. In fact, direct observation of livers of *W/W* fetuses 1 week after successful injection of normal hematopoietic cells in earlier studies has revealed that very few focal regions of hypercellularity, due to the normal donor cells, become established (7). If single-cell seeding is postulated to occur, then the maximum likelihood estimate of the number of cells seeded is 1.39 cells per mouse, and the fraction of animals seeded by a single cell is ≈46.2% (lower one-sided 90% confidence bound = 18.5%).

In our statistical analysis, we used a conditional Poisson distribution to describe the number of cells seeded and found that *P_b*, the chance that a mouse is seeded only by donor type B (BALB/c) is:

$$P_b = \exp(-m) \{ \exp(mp) - \sum [(mp)^r / r!] \} / \{ 1 - \exp(-m) \sum [m^r / r!] \},$$

where *m* is the unknown average number of cells seeded per mouse, and *p* is the fraction of these that are of B-type, also unknown. “Sum” indicates summation over *r* from zero to *k* – 1, where *k* is the minimum number of cells seeded. *k* is two in our statistical test; (details will be supplied to interested readers by the authors). *P_c*, the chance that only type C (C57BL/6) donor cells are seeded, is found similarly, and *P_d*, the chance that both donor types are seeded, is $1 - P_b - P_c$. The trinomial distribution,

$$[11 \text{ above } nb, nc, \text{ and } nd] (P_b^{**nb}) (P_c^{**nc}) (P_d^{**nd}),$$

gives the chance of observing any combination of outcomes as in Table 1, where *nb* = 2, *nc* = 6, and *nd* = 3. We found, for *k* = 2 cells, that the chance of this outcome or of less

Table 1. Frequency of engraftment of one or two strains of hematopoietic stem cells in mouse fetuses

Host strain*	No. with C57BL/6 donor	No. with BALB/c donor	No. with both donors	No. of cases
<i>W^f/W^f</i>	6	2	3	11
<i>W/W</i>	3	3	3	9
Totals	9	5	6	20

*The *W^f/W^f* host is on a C3H strain background; the *W/W* host is on a (C57BL/6-*Hbb^d/Hbb^d* × WH)*F*₁ background.

FIG. 2. RBC strain contributions (determined from hemoglobin and GPI markers) in six W^f/W^f recipients injected prenatally with a mixture of two normal strains of fetal liver cells. Bars: solid, C57BL/6 donor strain; open, BALB/c donor strain; stippled, host strain.

probable outcomes with the same sum $nb + nc$ or of outcomes in the same direction (larger $nb + nc$) did not exceed 10% over a wide range of the unknown parameters p and m . This range includes $0.3 < p < 0.57$ and all $m > 0$, and $0.25 < p < 0.725$ for $m > 0.66$. Since this range very likely includes the experimental value of p , the above conclusions are justified.

Hematopoiesis in adult irradiated or W -anemic mice has previously been found to be restored from a small number of stem cells in donor bone marrow, as estimated from unique chromosomal markers (2, 16–18) or clinical cure of the host's anemia by limiting dilutions of cells (19). Results in the present experiment support the conclusion that only a single stem cell from the small donor inoculum of fetal liver has

FIG. 3. In the same W^f/W^f host whose RBC are all of the C57BL/6 donor genotype (shown in Fig. 2A), granulocytes and lymphocytes are also of that strain. (Left) Two neutrophils with giant sudanophilic granules due to the *beige* marker in the C57BL/6-*bg^d/bg^d* donor. (Right) Serum from the host (center well) reacts positively with C57BL/6 allotype-specific antiserum (leftmost well), as does C57BL/6 control serum (upper left well).

become engrafted in some of the fetal hosts, especially in the W^f/W^f group; and that it is a THSC from which myeloid and lymphoid lineages have been monoclonally derived.

The experiment thus uniquely enables stem cell pedigrees to be examined in the normal developmental progression within individuals. [Data collected with a similar objective by using X chromosome-linked gene inactivation in heterozygous female mice (20) were later retracted (21).] In at least six of our animals out of seven containing two or three RBC genotypes for a sufficient period of observation, a complementary rise and fall was seen in the RBC subpopulations, with a periodicity of ≈ 20 –30 weeks. This occurred either in cells derived from the two donors (Fig. 2C; and two cases in W/W hosts, with data not shown), from one donor (C57BL/6) and the host (Fig. 2D and F), or, in a more complex interrelationship, in all three possible contributors (Fig. 2E). The complementary nature of the changes indicates physiologically regulated responsiveness. The RBC decline and resurgence and the temporary disappearance and reappearance of a donor strain in one W^f/W^f host (Fig. 2E) and in two W/W hosts (data not shown) suggest that the stem cell population is hierarchical. The hierarchy appears to consist of an actively proliferating but ephemeral compartment, whose differentiated products are more immediately visible, and a "hidden" reserve compartment. A waxing-and-waning wave of RBC then would represent a clone in the active compartment with a maximum life-span of 20–30 weeks. It then must be replaced from a smaller reserve of slowly dividing, more quiescent, and more long-lived cells that are the hematopoietic founder cells or their lineal descendants.

The hypothesis of clonal succession proposed by Kay (22) describes a model that our data seem to substantiate: small

numbers of primitive stem cells that are relatively quiescent are sequentially triggered to produce large amplifying clones. The stem cell population is thus heterogeneous, with an "age structure" of gradually decreasing self-renewal capacity and increasing proliferative activity, progressing toward differentiation (23). Various lines of evidence (reviewed in refs. 21 and 24) lend support to this view.

In our experiments, the cells from fetal liver (4, 5) or adult bone marrow (7) that are successfully engrafted in fetuses are operationally defined as THSC based on their high-renewal and broad-developmental potentials. Heterogeneity among them is indicated by variations in graft duration and by reduced self-renewal of cells from adult marrow relative to fetal liver; yet all are THSC. Those cells with higher renewal potential presumably come from the reserve compartment, and those with lower renewal, from the active compartment. If a normal THSC from the reserve compartment is singly seeded in a deficient fetus, under these demanding physiological circumstances it might realize a much greater renewal potential than ordinarily and supply the entire active compartment, thereby restoring normalcy for a full life-span. The W/W recipient in Fig. 4B, with all of its RBC from the BALB/c donor from 10 weeks onward, may exemplify imperfections in this arrangement: after repeated episodes of low hematocrit (below 20) at 18- to 24-week intervals, it died at 71 weeks with a hematocrit of 10%.

If somatic transfer of blood genes attains very high efficiency in fetal liver or bone marrow cells *in vitro*, single-cell engraftment of transformed THSC in mildly defective fetuses such as W^f/W^f would offer an optimal test for developmentally regulated expression in the appropriate lineage.

We thank R. Philip Custer for examination of tissues and Roy J. Riblet for analysis of immunoglobulin allotypes. This work was supported by grants HD-10646, CA-06927, and RR-05539 from the U.S. Public Health Service and by an appropriation from the Commonwealth of Pennsylvania.

1. Wu, A. M., Siminovitch, L., Till, J. E. & McCulloch, E. A. (1968) *J. Exp. Med.* 127, 455–464.
2. Abramson, S., Miller, R. G. & Phillips, R. A. (1977) *J. Exp. Med.* 145, 1567–1579.
3. Fialkow, P. J., Denman, A. M., Jacobson, R. J. & Lowenthal, M. N. (1978) *J. Clin. Invest.* 62, 815–823.
4. Fleischman, R. A. & Mintz, B. (1979) *Proc. Natl. Acad. Sci. USA* 76, 5736–5740.
5. Fleischman, R. A., Custer, R. P. & Mintz, B. (1982) *Cell* 30, 351–359.
6. Blanchet, J. P., Fleischman, R. A. & Mintz, B. (1982) *Dev. Genet.* 3, 197–205.

FIG. 4. RBC strain contributions in two W/W recipients injected prenatally with a mixture of C57BL/6 and BALB/c normal fetal liver cells. Strain identity of bars is as in Fig. 2.

7. Fleischman, R. A. & Mintz, B. (1984) *J. Exp. Med.* **159**, 731–745.
8. McCulloch, E. A., Siminovitch, L. & Till, J. E. (1964) *Science* **144**, 844–846.
9. Guénet, J.-L., Marchal, G., Milon, G., Tambourin, P. & Wendling, F. (1979) *J. Hered.* **70**, 9–12.
10. Murphy, E. D., Harrison, D. E. & Roths, J. B. (1973) *Transplantation* **15**, 526–530.
11. Whitney, J. B., III (1978) *Biochem. Genet.* **16**, 667–672.
12. Bücher, T., Bender, W., Fundele, R., Hofner, H. & Linke, I. (1980) *FEBS Lett.* **115**, 319–324.
13. Gearhart, J. D. & Mintz, B. (1972) *Dev. Biol.* **29**, 27–37.
14. Sheehan, H. L. & Storey, G. W. (1947) *J. Pathol. Bacteriol.* **59**, 336–337.
15. Russell, E. S. & Bernstein, S. E. (1966) in *Biology of the Laboratory Mouse*, ed. Green, E. L. (McGraw-Hill, New York), pp. 351–372.
16. Barnes, D. W. H., Ford, C. E., Gray, S. M. & Loutit, J. F. (1959) *Progr. Nucl. Energy Ser. 6* **2**, 1–10.
17. Micklem, H. S., Ford, C. E., Evans, E. P. & Ogden, D. A. (1975) *Cell Tissue Kinet.* **8**, 219–232.
18. Wallis, V. J., Leuchars, E., Chwalinski, S. & Davies, A. J. S. (1975) *Transplantation* **19**, 2–11.
19. Boggs, D. R., Boggs, S. S., Saxe, D. F. & Gress, L. A. (1982) *J. Clin. Invest.* **70**, 242–253.
20. Burton, D. I., Ansell, J. D., Gray, R. A. & Micklem, H. S. (1982) *Nature (London)* **298**, 562–563.
21. Micklem, H. S., Ansell, J. D., Wayman, J. E. & Forrester, L. (1983) in *Progress in Immunology V*, eds. Yamamura, Y. & Toda, T. (Academic, Tokyo), pp. 633–644.
22. Kay, H. E. M. (1965) *Lancet* **2**, 418–419.
23. Hellman, S., Botnick, L. E., Hannon, E. C. & Vignuelle, R. M. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 490–494.
24. Lajtha, L. G. (1979) *Differentiation* **14**, 23–33.