Engraftment of Immune-Deficient Mice with Human Hematopoietic Stem Cells

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A system in which immune-deficient mice are repopulated with cells from the human myeloid lineage, and that provides an in vivo stem cell assay for human hematopoietic cells is described. Generation of the chimeric human/immune-deficient (HID) mice was dependent on the use of immune-deficient *bg/nu/xid* mice. Infusion of these mice with human bone marrow gave rise to increases in human macrophage progenitors during more than 5 weeks of in vivo growth, indicating the seeding, proliferation, and differentiation of human stem cells. The human identity of the progenitors was confirmed by sequence analysis and their dependence on human growth factors. The creation of HID mice lays the foundation for establishing animal models for a wide variety of human hemopathies, from leukemia to infectious disease.

THE MATURE CELLS OF THE HEMATOpoietic system have a finite life-span and are being replenished continuously by the proliferation and differentiation of lineage specific progenitor cells derived from pluripotent hematopoietic stem cells (*1*). In the mouse we have information about the cells that comprise the stem cell hierarchy and the events that regulate this complex cell system because of the availability of both in vivo and in vitro assays for various stem and progenitor cells (*2*). In contrast, there are no in vivo assay systems for human pluripotent stem cells (*3*). Ideally, reconstitution of both the lymphoid and myeloid human lineages in the mouse would be useful. Mature human lymphoid cells can be engrafted in immune-deficient severe combined immune-deficient (SCID) mice after intraperitoneal injection of peripheral blood leukocytes (PBLs) (*4*), or after the intravenous injection of human fetal liver into mice previously surgically transplanted with human fetal thymus and lymph node (*5*). In both cases human T and B cells were found in the peripheral circulation after several weeks; the cells were present until ~9 weeks after the transplant. No evidence of myeloid engraftment was reported after fetal liver engrafment, although some macrophages were detected after PBL injection. Because mature lymphoid cells have a relatively long life-span and can proliferate, and because there are no colony assays for lymphoid progenitors, it is difficult to determine whether human stem cells have actually engrafted these mice. The myeloid system is easier to follow due to the availability of in vitro colony-forming assays for a variety of committed and multipotential progenitor cell types (*1*). We now report the development of an in vivo human hematopoietic stem cell assay that was achieved by engraftment of myeloid cells into immune-deficient mice with normal human bone marrow.

There are at least two major barriers that could prevent the growth of transplanted human bone marrow in irradiated recipient mice: the presence of NK cells and lymphokine-activated killer cells (LAK), and the absence of human hematopoietic growth factors. Lethally irradiated mice still possess enough immune function to reject the foreign cells. Even immune-deficient mice, which lack functional T and B lymphocytes, such as the SCID and the nude have high levels of NK and LAK activity (*6, 7*). To overcome the cytotoxic activity we used *bg/nu/xid* mice. The nude (*nu*) mutation renders the animals athymic, the beige (*bg*) mutation reduces the number of NK cells, and the *xid* mutation reduces the number of lymphokine activated killer cells (LAK), an activity important in the host response to foreign cells (*8*).

Several hematopoietic growth factors are species specific; for example, murine IL-3 does not stimulate the growth of human multipotent progenitor cells and human IL-3 has no effect on murine cells (*9*). Therefore, in our initial experiments human IL-3 and GM-CSF were continuously infused into the *bg/nu/xid* mice with the use of an osmotic minipump. The pumps were im-

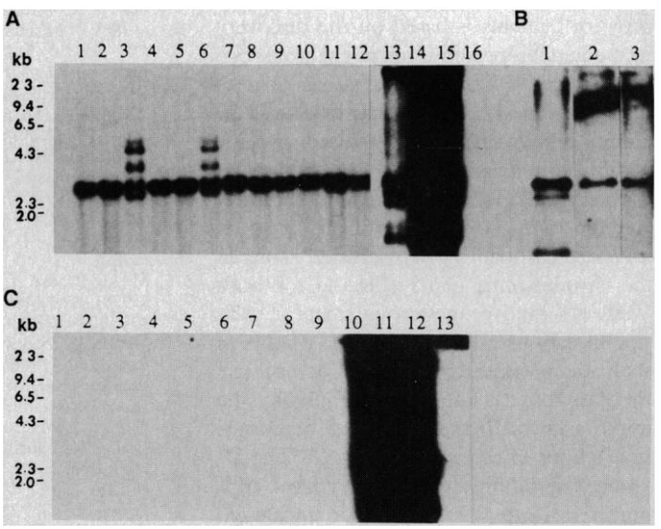

Fig. 1. DNA analysis of bone marrow and spleen from animals 14 days after reconstitution with normal human bone marrow. Mice were generated as described (*21*). The DNA was extracted from bone marrow and spleen (after sectioning the spleen into five pieces), digested with Eco R1, blotted according to standard procedures, and probed with p17H8, a human α-satellite probe specific for sequences on chromosome 17 (*11*). Eco R1 digestion of human DNA produces a characteristic 2.7-kb band; in addition, one-third of the population has polymorphisms that give rise to a series of lower molecular weight minor bands (*11*). The autoradiograms were exposed for varying lengths of time and analyzed with a Biorad video densitometer to quantitate band intensity. (**A**) DNA (5 μg) from two HID mice 14 days (lanes 1 to 6) and 17 days (lanes 7 to 12) after transplantation with human bone marrow. Lanes 1 and 7, bone marrow; lanes 2 to 6 and 8 to 12, spleen pieces. Control mixtures of human and mouse DNA (10 μg): lane 13, 1% human; lane 14, 30% human; lane 15, 50% human; lane 16, mouse DNA. The ethidium bromide–stained gel prior to Southern transfer indicated that the DNA from lanes 3 and 6 was partially digested. Other gels where these samples were completely digested produced a single band. Lanes 1 to 12 and 16 were exposed for 36 hours while lanes 13 to 15 for 6 hours. After correction for exposure differences, the band intensity in the DNA from HID mice represented 0.3% human DNA. (**B**) DNA (5 μg) from an HID mouse 14 days after transplantation with human bone marrow. Lane 1 contains human DNA (5 μg) alone (45-minute exposure), while lanes 2 and 3 are from the HID mouse spleen and bone marrow (16.5-hour exposure). Correcting for exposure differences, the band intensity in the DNA from HID mice represented 2% to 3% human DNA. (**C**) DNA analysis of spleen DNA from CBA/J mice 7 to 10 days after transplantation with human bone marrow. All mice were treated as described except the radiation dose was raised to 950 cGy in an attempt to improve engraftment. Lanes 1 and 2, spleen 10 days after transplantation; lanes 3 to 5, spleen 7 days after transplantation; lanes 6 to 9, spleen 7 days after transplantation; lanes 10 to 12, mixtures of human and mouse DNA. Lane 10, 50% human; lane 11, 30% human; lane 12, 1% human; and lane 13, mouse DNA. Exposure was for 36 hours. Molecular size markers indicated in kilobases.

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planted 1 day before irradiation and intravenous injection of human bone marrow.

Successful transplantation of normal mouse bone marrow into immune-deficient mice requires irradiation of the recipient mice (*10*). From preliminary experiments we found that equivalent engraftment was obtained in recipients receiving 400 or 800 cGy. Since many animals died 14 days after 800 cGy, all subsequent experiments were carried out using 400 cGy to examine engraftment beyond 14 days. Human bone marrow cells (10^7) were injected intravenously into irradiated CBA/J and *bg/nu/xid* recipients who carried minipumps. After 14 days DNA from the bone marrow and spleen was analyzed by Southern blot to quantitate the proportion of human cells in the hematopoietic tissues of the recipient. The probe was an α-satellite sequence specific for human chromosome 17 that does not hybridize with mouse DNA (*11*). Human DNA was detected in the bone marrow and spleen from transplanted *bg/nu/xid* animals (Fig. 1). The uniform intensity of the bands in the bone marrow and spleen sections suggests that the donor cells seeded these tissues with an even distribution rather than as discrete foci. Quantitative analysis of band intensities at different exposures followed by densitometry indicated that human cells accounted for approximately 0.3 to 3.0% of the spleen and bone marrow. No evidence of engraftment was observed in the CBA/J mice (Fig. 1C), confirming the importance of *bg/nu/xid* mice as recipients.

We next determined whether progenitor cells had been engrafted into the HID mice. Single cell suspensions of bone marrow and spleen were plated in in vitro colony assays optimized for the growth of human granulocyte-macrophage progenitor cells (human granulocyte-macrophage colony-forming unit; hCFU-GM) (Table 1). Mixing experiments indicated that normal CBA/J or *bg/nu/xid* bone marrow produced very low numbers of colonies that generally die by day 8 in the presence of human growth factors [(*12*) Table 1]. In contrast, HID mice contained significant numbers of progenitors that produced large diffuse colonies that were viable for more than 12 days in culture (Fig. 2A). Most cells from individual colonies had typical macrophage morphology with a high cytoplasm/nucleus ratio and foamy cytoplasm (Fig. 2B), and some colonies contained granulocytic cells indicating that they arose from CFU-GM or macrophage (CFU-M) progenitor cell types. Our culture conditions were not specially optimized for erythroid progenitors, however BFU-E (approximately 2% of the number of hCFU-M) were detected in four HID mice after 4 weeks of engraftment. Cultures from CBA/J mice that received human bone marrow did not contain any colonies (Table 1). SCID mice transplanted using the same protocol contained low numbers of human progenitors (Table 1), indicating that *bg/nu/xid* mice were a better environment for stem cell engraftment. While the reason for this is not clear, it may be due to high levels of NK activity in SCID mice, while *bg/nu/xid* mice are mildly deficient in NK activity and severely deficient in LAK activity. Because engraftment of mature human lymphoid cells has been demonstrated in SCID mice (*4, 5*) this suggests fundamental differences in the requirements of myeloid stem cells and mature lymphoid cells for engraftment of immune-deficient mice.

To obtain independent evidence that the colonies derived from the HID mice were human, we used the polymerase chain reaction (PCR) to detect the presence of human chromosome 17 α-satellite DNA sequences in single hematopoietic colonies (*13*). The human α-satellite sequences are composed of a family of closely related 171-bp tandem repeats. Oligonucleotide primers specific for the 5′ and 3′ ends of the consensus repeat sequence were used. The PCR amplification of normal human DNA results in a complex pattern of bands, because different numbers of repeats act as templates in the PCR reaction (Fig. 3). The human identity of these bands was confirmed by Southern blot analysis of the amplified products using the α-satellite probe. All of the colonies from two HID mice transplanted with human bone marrow had amplified bands, while no amplification products were seen in the negative control that was done with an excess of mouse DNA of up to 10^4 times, to ensure there was no background amplification (*14*). The PCR analysis of small colonies (with less DNA) had only faint bands (Fig. 3, lanes 3 and 4). Two samples from the spleens of CBA/J mice that had received human bone marrow showed no detectable amplification, supporting our previous conclusion that no engraftment occurred in these animals. Taken together, the molecular identification of human DNA and their human growth factor dependence established the colonies as human.

If the number of progenitors detected in HID mice arose by differentiation from more primitive precursors, then the number of hCFU-M should increase after transplantation and reach a steady state that is maintained for an extended period of time. We

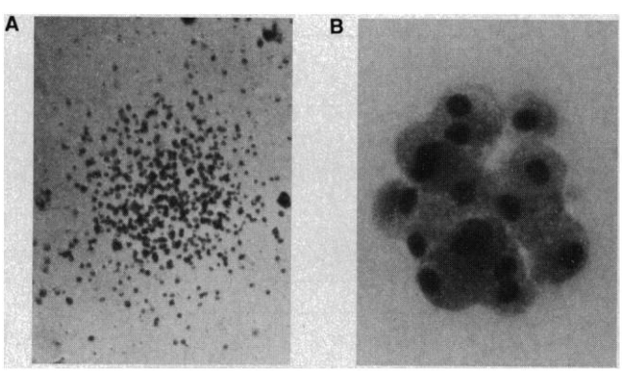

Fig. 2. Colonies derived from methylcellulose cultures of bone marrow from *bg/nu/xid* mice previously engrafted with human bone marrow. (**A**) Typical colony at day 11 growing in methylcellulose culture under the conditions described in the legend to Table 1. (**B**) Wright-stained cells from individual colonies. These cells have characteristic macrophage morphology.

Table 1. In vitro colony formation of bone marrow from normal and HID mice. The method of engrafting the mice was as in Fig. 1. All mice received 400 cGy irradiation except as noted. Bone marrow was aspirated from both femurs and tibia, counted, and plated in methylcellulose cultures. Briefly, 2×10^5 cells were plated in methylcellulose, human plasma (30%), erythropoietin (1 U/ml), and phytohemagglutinin-stimulated leukocyte conditioned medium (PHA-LCM, 10%) (*18, 19*). The total bone marrow counts were calculated assuming that both femurs and tibia account for 19% of the total mouse bone marrow (*20*). The data below are from single animals and are representative of the number of animals shown in parenthesis.

Mouse strain (*n*)	Pump with factors	Human bone marrow	Total bone marrow cells per mouse	hCFU-M† (per 2×10^5 cells plated)	Total hCFU-M per mouse
CBA/J* (10)	+	−	3.1×10^8	0	0
CBA/J (4)	+	+	2.4×10^8	0	0
bg/nu/xid (12)	+	+	3.2×10^8	175 ± 17	2.8×10^5
bg/nu/xid (7)	−	+	3.1×10^8	131 ± 64	2.0×10^5
SCID (2)	+	+	8×10^6	9 ± 5	360

*Not irradiated; the ten control mice tested included three *bg/nu/xid* mice (*12*). †Mean ± SD of more than four plates.

compared the total number of hCFU-M in the bone marrow and spleen of *bg/nu/xid* recipients (from five independent experiments) sacrificed between days 5 and 35 after transplantation with bone marrow from different donors (Fig. 4). The animals received between 5×10^6 and 10×10^6 normal human bone marrow cells. The "0" time point indicates the total number of hCFU-GM transplanted. The total hCFU-M in the bone marrow of the animals increased rapidly, with over 40 times more hCFU-M at day 14 than the input number. Animals sacrificed at 5 weeks still maintained the same level of engraftment. The spleen was also engrafted for up to 5 weeks, although the kinetics followed a different pattern. There appeared to be delayed seeding of the spleen because only low levels of hCFU-M were detected at day 5, with increases by day 14. The seeding efficiency of murine transplanted cells into hematopoietic tissues is considerably less than the input number [<10% (*15*)]. This implies that the actual number of human progenitors seeding the bone marrow or spleen is less than the input number indicated in the figure, therefore the calculations of the population increase are only a minimum estimate of the true value.

We also determined the necessity for exogenously supplied human growth factor. Animals were transplanted according to the protocols described earlier except they received saline or no minipump (Table 1 and Fig. 4). These animals maintained similar levels of hCFU-M for as long as animals receiving the factors, indicating that delivery of additional human IL-3 and GM-CSF was not required for the production of hCFU-M. This suggests that either the human stem cells are responding to murine growth factors or microenvironment, the human cells are producing their own growth factors, or both.

The results presented above demonstrate the successful engraftment of lightly irradiated *bg/nu/xid* mice after intravenous infusion of normal adult human bone marrow. The engraftment is rapid and a large proportion of the engrafted cells are progenitors capable of giving rise to macrophage colonies in vitro. The kinetics indicate that large numbers of hCFU-M can be produced in the bone marrow and to a lesser extent in the spleen. This level of engraftment was maintained for the 5 weeks that we have followed the HID mice. Since the progenitors assayed in vitro have little self-renewal potential (*1*), this large sustained increase implies that an earlier progenitor or stem cell had seeded the bone marrow and spleen and was responsible for producing the hCFU-M. These experiments raise many questions regarding the cell types participating in the reconstitution process. Although the nature of the cell giving rise to the hCFU-M is unknown, these data provide a foundation for the first in vivo human stem cell assay in a small animal. While this is not a colony assay, it is similar to the murine long-term repopulation assays for pluripotent stem cells (*1, 2*). It has been possible to follow individual clones of genetically engineered murine stem cells as they repopulate recipient mice by using the random retrovirus integration site as a molecular marker (*16, 17*). Using protocols that permit high efficiencies of gene transfer into human hematopoietic cells (*18*), it should now be possible to carry out a similar clonal analysis of the organization of the human hematopoietic system (*16, 17*). The HID mice will also be a valuable model in which to test gene transfer and long-term expression in human hematopoietic cells as a prelude to human gene therapy trials. Furthermore, the HID mice may prove to be an important system (i) to develop models of various human hemopathies, including leukemia, sickle cell anemia, and thalassemia; (ii) to develop assays for growth factors and chemotherapeutic agents; and finally (iii) for the study of human infectious diseases that affect the hematopoietic system.

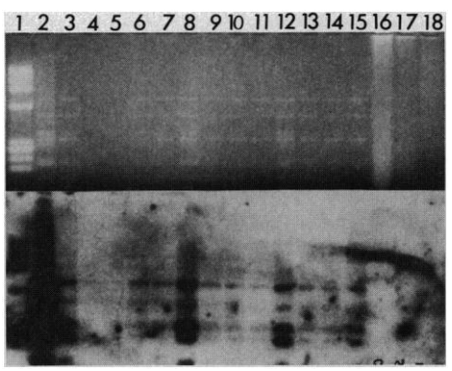

Fig. 3. Identification by PCR analysis of human DNA in individual colonies from methylcellulose cultures from HID mice. Single colonies were picked from methylcellulose cultures that had been established from HID bone marrow, were lysed, treated with proteinase K, and subjected to 30 rounds of PCR. The sequences of the two oligonucleotide primers were: sense, ACGATTCTCAGAAACTTCTTTGTGAT; antisense, TTTTATATGAAGATATTCCC. (**Top**) An ethidium bromide–stained agarose gel after electrophoresis of one-tenth of the product of the PCR reaction. (**Bottom**) Southern blot of the agarose gel, probed with the p17H8 probe as described in Fig. 1. Lane 1, ϕX174; lane 2, 500 ng of human DNA; lanes 3 to 15, individual hematopoietic colonies from methylcellulose cultures derived from two HID mice; lanes 16 and 17, spleen DNA from the CBA/J mice described in Fig. 1; lane 18, mouse DNA.

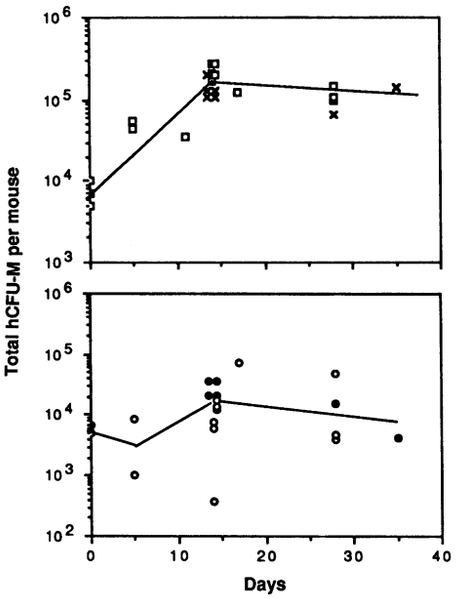

Fig. 4. Increase in the number of the hCFU-M from HID mice with time of reconstitution. The number of hCFU-M were measured in mice that had been reconstituted for the length of time indicated. Total hCFU-M were calculated as described in Table 1. Each point represents the number of hCFU-M from the bone marrow (**top**) and spleen (**bottom**) of one animal reconstituted for the length of time indicated. The lines were fitted by eye to indicate the trend in the data. These data represent five independent experiments with the use of bone marrow from five different human donors. The animals denoted by the (x) or the (●) contained a pump with saline.

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12. Analysis of data on over 45 plates from seven CBA/J and three *bg/nu/xid* mice, with or without minipumps, indicated that on average less than ten colonies grew in cultures plated in PHA-LCM and virtually no colonies arose in plates with human granulocyte-macrophage colony-stimulating factor (hGM-CSF), human interleukin-3 (hIL-3), or both. If a small amount of breakthrough did occur, this number was subtracted from the experimental group. To rule out the possibility that the presence of human cells permitted the growth of murine progenitors mixing experiments were carried out. Various combinations of human:mouse bone marrow cells were plated in (PHA-LCM), (hGM-CSF), (hIL-3), or hGM-CSF + hIL-3. A typical experiment using PHA-LCM was: 100% human:0% mouse, 165 colonies; 50:50, 80 colonies; 10:90, 17

colonies; 1:99, 8 colonies; and 0:100, 6 colonies.
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24. The animal experiments were approved by the Animal Care Committee at our institution. Human bone marrow was obtained after informed consent from normal donors with the use of procedures approved by the Human Experimentation Committee at our institution. Supported by grants from the Medical Research Council of Canada and the National Cancer Institute of Canada (J.E.D.) and an MRC postdoctoral fellowship (S.K.-R.). We thank S. Clark for hematopoietic growth factors, C. Hansen for the *bg/nu/xid* breeding pairs, and H. Willard for the human α-satellite probe. We also thank B. Murdoch and M. Doedens for excellent technical assistance, P. Laneuville for the original idea and human bone marrow, R. Phillips for his advice and support, and L. Siminovitch, R. Worton, A. Bernstein, and N. Iscove for critically reading the manuscript.

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