Antigen CD34$^+$ Marrow Cells Engraft Lethally Irradiated Baboons

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Abstract

The CD34 antigen is present on 1–4% of human marrow cells including virtually all hematopoietic progenitors detected by in vitro assays. Since the anti–CD34 monoclonal antibody 12-8 reacts with a similar marrow population in baboons, it was possible to test whether this antigen is expressed by stem cells responsible for hematopoietic reconstitution in vivo. CD34$^+$ cells were enriched from marrows of five baboons using avidin-biotin immunoadsorption. After lethal irradiation, the five animals were given 15–27 $\times$ 10^6 autologous marrow cells (3.2–4.4 $\times$ 10^6 cells/kg) containing 65–91% CD34$^+$ cells. All animals achieved granulocyte counts > 1,000/mm^3 and platelet counts > 20 $\times$ 10^3/mm^3 by 13–24 d posttransplant and subsequently developed normal peripheral blood counts. Two additional animals received 184 and 285 $\times$ 10^6 marrow cells/kg depleted of CD34$^+$ cells. One animal died at day 29 without engraftment, while the other had pancytopenia for > 100 d posttransplant. The data suggest that stem cells responsible for hematopoietic reconstitution are CD34$^+$.

Introduction

Studies of human hematopoietic stem cells have been limited by the inability to identify and isolate these progenitor cells from marrow. Monoclonal antibody technology has made it possible to characterize cell surface antigens expressed by human hematopoietic progenitors and their progeny (1, 2). The CD34 antigen identified by antibodies 12-8, MY-10, BI-3C5, and ICH3 is of particular interest because it is primarily found on immature hematopoietic elements in human marrow (3–6). Nearly all colony-forming progenitors, granulocyte-macrophage colony-forming units (CFU-GM)[1], erythroid burst-forming units (BFU-E), and multipotential colony-forming units (CFU-MIX), detectable with in vitro assays express the CD34 antigen. Antibody 12-8 also identifies the precursors of colony-forming cells in long-term marrow culture (5). In nonhuman primates, this antibody also reacts with 1–4% of marrow cells that contain virtually all hematopoietic colony-forming progenitors (7). To test whether CD34$^+$ marrow cells were capable of restoring hematopoiesis in vivo, lethally irradiated baboons were transplanted with autologous CD34$^+$ marrow cells enriched by avidin-biotin immunoadsorption chromatography (8–11).

Methods

Baboons (Papio cynocephalus) and animal treatment

Baboons that were born at the University of Washington Regional Primate Center were placed in isolation and dewormed prior to use. The animals weighed 3.9–5.4 kg and ranged 1–2 yr in age. Research was conducted at the Primate Center under conditions that met National Institutes of Health standards as stated in the Guide for the Care and Use of Laboratory Animals (DHEW Publication No. NIH 85-23, 1985), Institute of Laboratory Animal Resources recommendations, and American Association for Accreditation of Laboratory Animal Care accreditation standards for animals of this species. A central venous catheter was placed in all animals. Marrow was obtained by aspiration of both femora and processed using previously established procedures (12). After marrow aspiration, all animals were given 9.2 Gy of total body irradiation as a single exposure delivered from two opposing ^{60}Co sources at a rate of 7 cGy/min.

Peripheral blood counts were determined daily until normal counts were achieved and then weekly thereafter. Serum electrolytes as well as other laboratory tests and X-rays were performed as clinically indicated. Parenteral fluid and electrolytes were given based on clinical status (e.g., vomiting and diarrhea) and laboratory values. Prophylactic antibiotics (piperacillin 100 mg/kg t.i.d. and gentamicin 1.7 mg/kg t.i.d.) were administered intravenously starting immediately posttransplant and continued until animals achieved a granulocyte count > 500/mm^3. The animals were given whole blood transfusions from random baboons for treatment of severe and life-threatening anemia and thrombocytopenia. All blood products used for transfusion were irradiated in vitro (15 Gy). Gross and histologic postmortem examinations were carried out on all animals that died.

Antibodies and immunofluorescence staining reagents

Antibody 12-8 (murine IgM isotype) was partially purified from ascites fluid using boric acid precipitation (13). Affinity-purified goat anti-mouse IgM antiserum was obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL) and biotinylated as previously described (9). The fluorescein isothiocyanate conjugate of avidin (avidin-FITC) was obtained from Vector Laboratories (Burlingame, CA).

Production of avidin-Biogel

Avidin obtained from Calbiochem-Behring Corp. (San Diego, CA) was conjugated to Biogel P-30 (Bio-Rad Laboratories, Richmond, CA) using a minor modification of a previously published procedure (9, 10). To make the gel used for positive cell selection, carboxylated Biogel P-30 was treated successively with 10 μg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC-HCl) and 100 μg of avidin per ml of gel. To make the gel used for depletion, Biogel P-30 was treated successively with 20 μg/ml EDC-HCl and 1 mg of avidin for each ml of gel.

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1. *Abbreviations used in this paper:* BFU-E, erythroid burst-forming unit; CFU-GM, granulocyte-macrophage colony-forming unit; CFU-MIX, multipotential colony-forming unit.

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Coupling of avidin to the gel was nearly quantitative under these conditions so that each milliliter of gel used for positive selection contained 100 μg of avidin and each ml of gel used for depletion contained 1 mg of avidin.

Antibody treatment, column separation, and FACS sorting of column-separated cells

Antibody treatment. Nucleated marrow cells (50×10^6/ml) obtained from a buffy coat preparation were incubated with antibody 12-8 (50 μg/ml) in phosphate-buffered saline (PBS) with 1% bovine serum albumin (PBS/BSA) for 30 min at 4°C. The cells were washed twice and then incubated at the same cell concentration in PBS/BSA with 1 μg/ml of biotinylated goat anti-mouse IgM antiserum for an additional 30 min at 4°C. The cells were again washed twice and adjusted to a concentration of $75–100 \times 10^6$ cells/ml in PBS/BSA for column treatment.

Isolation of CD34$^+$ cells. The antibody-treated cells were passed over a Chromaflex 15 × 2.5-cm column (Kontes Co., Vineland, NJ) containing 20 ml avidin-Biogel (100 μg/ml avidin) at a flow rate of 6–12 ml/min until a total volume of ~ 100 ml had been collected. ~ 50 ml of PBS was then passed through the gel at the same flow rate to wash out BSA. The adherent cells were dislodged by mechanical agitation with a 10-ml pipette until a total volume of 100 ml had been collected. The recovered adherent cells were directly infused in three animals.

In two animals, recovered adherent cells were further separated by FACS prior to infusion. In this procedure, the adherent cells at a concentration of 10^7 cells/ml were incubated with a 1:100 dilution of avidin-FITC in PBS/BSA for 30 min at 4°C. The cells were washed twice and then separated by flow microfluorimetric sorting on a FACS IV (Becton Dickinson Immunocytometry Systems, Mountain View, CA) as previously described (5). The cells with a level of fluorescence intensity > 98% of unlabeled cells were collected and infused into these two animals.

Depletion of CD34$^+$ cells. Nucleated marrow cells were treated successively with antibody 12-8 and biotinylated goat anti-mouse IgM antiserum as described above. These cells at a concentration of 75×10^6 cells/ml were passed over a Chromaflex 25 × 2.5-cm column containing 100 ml of avidin-Biogel (1 mg/ml avidin) at a flow rate of 1 ml/min. The nonadherent cells that washed through the column were collected and infused.

Immunofluorescence studies

The percentage of cells reactive with antibody 12-8 was determined by immunofluorescence staining and flow microfluorimetric analysis by using an indirect immunofluorescence procedure (9, 10). Briefly, 5×10^5 cells were incubated with a 1:100 dilution of avidin-FITC in PBS/BSA with 0.1% sodium azide for 20 min at 4°C. The stained cells were washed before and after exposure to hemolytic buffer and then analyzed with a FACS IV and computer 440 (Becton Dickinson Immunocytometry Systems). The unseparated and adsorbed cells labeled with antibody 12-8 and biotinylated goat anti-mouse IgM antiserum were stained with avidin-FITC. As a negative control, the unlabeled marrow cells were stained with avidin-FITC or incubated successively with a nonreactive, control IgM antibody and biotinylated goat anti-mouse IgM antiserum and stained with avidin-FITC. The percentage of CD34$^+$ cells was determined by subtracting the percentage of cells positively staining that were labeled with the control reagents from the percentage of cells positively staining after labeling with antibody 12-8 and biotinylated goat anti-mouse IgM antiserum.

Colony-forming cell assays

Cells from unseparated and separated baboon marrow were cultured at 10^4 to 10^5 cells per 35-mm culture dish in semisolid agar culture medium. Medium consisted of Iscove's modified Dulbecco's medium (Gibco, Grand Island, NY) supplemented with 20% fetal bovine serum (Hyclone, Logan, UT), 3 IU/ml human urinary erythropoietin (Terry Fox Cancer Center, Vancouver, BC, Canada), 10% human placental conditioned medium, 10^{-4} M 2-mercaptoethanol, and 0.3% (wt/vol) agar (Seapaque, FMC Corp, Rockland, ME). Medium conditioned with human placental tissue was prepared as a crude source of colony-stimulating activities by the method of Schlunk and Schleyer (14). Cultures were incubated at 37°C, 5% CO_2 in a humidified incubator and scored for different colony types after 14–16 d using an inverted microscope.

Results

Transplantation of enriched CD34$^+$ cells. Using antibody 12-8, cells expressing the CD34 antigen were positively selected from the marrows ($0.8–3.6 \times 10^9$ cells) of five baboons (Table I). In three experiments, $20–27 \times 10^6$ cells adherent to the column were recovered. These cells were 65–81% CD34$^+$ and represented 1.6–2.5% of the starting cell number applied to the column. Fig. 1 shows an example of the enrichment of CD34$^+$ cells in the column adherent population compared with the unseparated marrow cells from one of the baboons. In two additional experiments, recovered column-adherent cells were separated by flow microfluorimetric sorting to further enrich CD34$^+$ cells. This yielded 19 and 15×10^6 cells that were 85 and 91% CD34$^+$ and accounted for 0.5% and 0.6% of the starting number of marrow cells, respectively. The committed hematopoietic progenitors including CFU-GM, BFU-E, and CFU-MIX were enriched in the positively selected cell populations (Table II). The CD34$^-$ cells isolated by flow microfluorimetric sorting from the column-adherent population contained few colony-forming cells.

After 9.2 Gy of total body irradiation, the five animals received $3.2–4.4 \times 10^6$ selected cells/kg ($2.7–3.5 \times 10^6$ CD34$^+$ cells/kg). All animals achieved granulocyte counts > 1,000/mm^3 and platelet counts > 20×10^3/mm^3 by 13–24 d, and received their last whole-blood transfusions 8–21 d posttransplant (Table I). Marrow aspirates and biopsies obtained 3–4 wk after transplantation showed normal marrow cellularity and the presence of all hematopoietic lineages in all animals. Fig. 2 shows that the temporal pattern of recovery of leukocytes and platelets in these baboons was similar to that observed in control animals given unprocessed marrow (see below).

Transplantation of marrow depleted of CD34$^+$ cells. Two animals received 0.9 and 1.2×10^9 cells (184 and 285×10^6 cells/kg, respectively) depleted of CD34$^+$ cells by immunoadsorption with antibody 12-8 (Table I). This treatment resulted in a 1–2-log reduction in detectable colony-forming cells (Table II). One animal died at day 29 without evidence of engraftment. The second animal demonstrated marrow aplasia for > 2 mo after transplantation. This animal had a granulocyte count < 100, platelet count < 20,000, and continued to require transfusions for > 100 d posttransplant. This baboon continued to have severe myeloid hypoplasia documented on serial marrow examinations until its death on day 227.

Control animals. Three animals received 206, 218, and 270 $\times 10^6$ cells/kg of unprocessed marrow after total body irradiation. Two animals engrafted with a temporal pattern of hematologic recovery similar to that of the animals transplanted with CD34$^+$ cells (Table I and Fig. 2). The third animal developed severe gastrointestinal bleeding and died at day 19 posttransplant with a total white blood cell count of 1,500, a granulocyte count of 172 (day 15), a platelet count of 7,000/mm^3,

Table I. Baboon Marrow Transplants: Cell Separation and Engraftment Data

	Baboon	Original cells	Separation procedure	Cells infused			Day posttransplant			Survival
				Total		CD34+	Neutrophils	Platelets	Last transfusion	
		$\times 10^6$		$\times 10^6$	$\times 10^6$/kg	%	>1,000/mm³	>20,000/mm³		d
Enriched	1	1,500	Column	27	4.1	65	13	14	13	103
	2	1,500	Column	24	4.4	81	20	18	17	110
	3	800	Column	20	4.0	68	23	13	8	93
	4	3,600	Column → FACS	19	3.9	85	21	19	12	413+
	5	2,400	Column → FACS	15	3.2	91	20	24	21	184+
Depleted	6	1,300	Depletion	1,200	285.0	<1	177	126	112	227
	7	1,400	Depletion	920	184.0	<1	NR	NR	NR	29
Unseparated	8	950	None	950	206.0	ND	17	20	16	57
	9	1,200	None	1,200	218.0	ND	20	18	11	29+
No marrow	10	0	None	0	—	—	NR	NR	NR	17

Cell separation procedures and engraftment data are summarized for 10 animals. Five animals received CD34+ enriched cells isolated by column immunoadsorption alone (baboons 1–3) or column immunoadsorption followed by flow microfluorimetric sorting (FACS, baboons 4 and 5). Baboons 6 and 7 received marrow depleted of CD34+ cells by column immunoadsorption. Baboons 8 and 9 were controls and received unmodified whole marrow. Baboon 10 was given no marrow and served as radiation control. The table shows the day after transplant when each animal achieved neutrophil counts > 1,000/mm³, untransfused platelet count > 20,000/mm³, and the last day that animals required red cell transfusions. ND, not done; NR, not reached.

and marrow that showed trilineage engraftment at autopsy. One animal that was irradiated and given no marrow died at day 17 with marrow aplasia at autopsy (Table I).

Posttransplant syndrome and long-term survival. All animals surviving greater than 1 mo developed a syndrome characterized by atypical lymphocytosis, hemolytic anemia with elevated reticulocyte count, thrombocytopenia, and lymphadenopathy. During this illness, animals that were transplanted with CD34+ cells had histologically normal marrows as determined by biopsy and in vitro assays demonstrated the presence of marrow colony-forming cells. A control animal given unprocessed marrow and the prolonged survivor transplanted with marrow depleted of CD34+ cells also developed this syndrome. This syndrome had its onset between 1 and 4 mo after transplant. Five animals died with interstitial pneumonia and/or encephalitis during the illness, and two animals transplanted with CD34+ cells recovered. One control animal, given unprocessed marrow, is < 1 mo posttransplant and will need longer follow-up to determine if it also develops this syndrome. The etiology of the illness has not been established although a viral agent is suspected. Bacteria and fungi have not been found nor have cytomegalovirus, Epstein-Barr virus, herpes viruses, or retroviruses been isolated from these animals.

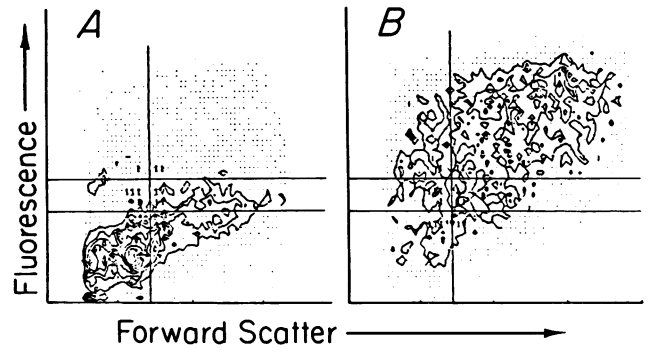

Figure 1. Marrow cells from second baboon before and after column separation. Unseparated marrow cells labeled with antibody 12-8 and biotinylated goat anti-mouse IgM antiserum (2A), and antibody-labeled adherent cells recovered from the column (2B) were stained with avidin-FITC and analyzed by flow microfluorimetry. The unseparated marrow cells were 1.5% CD34+, while the recovered adherent cells were 81% CD34+.

Discussion

Several monoclonal antibodies have been developed that identify the CD34 antigen expressed by early human hematopoietic progenitors detected by in vitro assays (3–6). One of these antibodies, 12-8, has the unique property of recognizing a distinct epitope that is also present on a similar marrow population in non-human primates (7). In the present study, highly enriched populations of CD34+ cells isolated from baboon marrow were capable of completely restoring hematopoiesis when transplanted into lethally irradiated animals. The rate of engraftment in these animals was similar to that of control animals transplanted with unseparated marrow and was similar to that reported in previous studies of autografts in lethally irradiated nonhuman primates (15, 16). Two animals received CD34+ cells that were further enriched by flow microfluorimetric sorting after immunoadsorption and demonstrated prompt engraftment and complete hematologic recovery. Therefore, the CD34− cells contaminating the adsorbed cell population are unlikely to have played a role in restoring hematopoiesis. The rapid and durable engraftment for more than 6 mo and 1 yr in these two animals suggests that the stem cells that produce hematopoietic reconstitution are contained

Table II. Colony-forming Cells Detected after Enrichment or Depletion of CD34⁺ Cells

	Baboon	Cell fraction	CFU-GM	BFU-E	CFU-MIX
				Colonies/10⁵ cells	
Enrichment	1	Unseparated	94±13	22±7	7±5
		Adherent	2500±280	500±80	200±44
		Nonadherent	40±6	9±3	2±1
	2	Unseparated	101±15	27±5	2±1
		Adherent	1356±101	197±53	123±29
		Nonadherent	37±5	13±2	1±0
	3	Unseparated	39±9	13±5	1±1
		Adherent	2246±346	1226±300	400±73
		Nonadherent	13±5	3±1	1±1
	4	Unseparated	81±12	60±9	7±4
		Adherent alone	1389±97	853±64	161±17
		Adherent → FACS CD34⁺	1966±217	1506±129	216±25
		Adherent → FACS CD34⁻	5±1	15±4	0±0
		Nonadherent	22±9	50±6	2±1
Depletion	6	Unseparated	80±7	40±5	5±1
		Adherent	1008±63	240±34	56±7
		Nonadherent	<1	2±1	<1
	7	Unseparated	56±9	36±3	4±4
		Adherent	309±34	118±10	25±6
		Nonadherent	4±1	15±2	0±0

Column immunoadsorption was used to either enrich (baboons 1–4) or deplete (baboons 6 and 7) CD34⁺ marrow cells. Marrow cells before column treatment (unseparated), cells bound to the column (adherent), and cells that passed through the column (nonadherent) were assayed for in vitro colony-forming progenitors (see Methods). In baboon 4, the adherent cells were further separated by flow microfluorimetric sorting (FACS) into CD34⁺ and CD34⁻ fractions. Data were not available for baboon 5 due to technical problems with the assays.

within the CD34⁺ cell population. Of two animals given marrow depleted of CD34⁺ cells, one died without evidence of engraftment, while the second animal demonstrated marrow aplasia for over 2 mo after transplant, pancytopenia for > 3 mo posttransplant, and continued to show marked hypoplasia of myeloid elements on serial marrow examinations until its death. The data suggest that CD34⁺ cells are both necessary and sufficient for complete hematopoietic reconstitution in vivo.

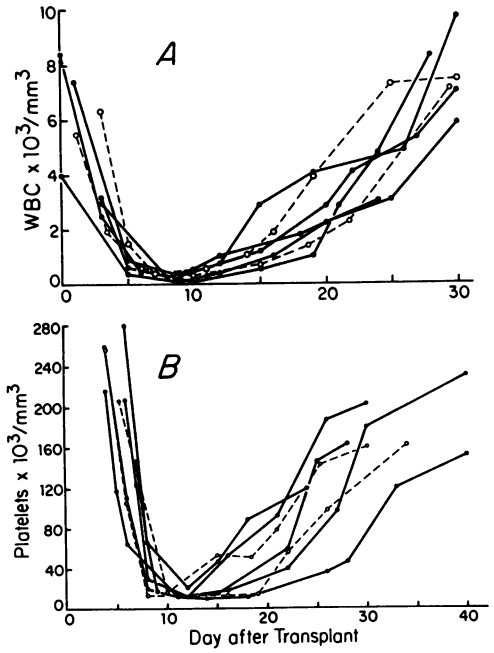

Figure 2. Total leukocyte count (2A), measured serially posttransplant in baboons transplanted with CD34⁺ cells (●) or whole marrow (○). Platelet count (2B) measured serially post-transplant in baboons transplanted with CD34⁺ cells (●) or whole marrow (○).

The possible contribution of endogenous radioresistant stem cells to hematopoietic reconstitution cannot be determined from these studies. The radiation dose used in these experiments resulted in lethal marrow aplasia in a control animal and has been shown previously to produce lethal marrow aplasia in baboons (17, 18). However, it is possible that transfused CD34⁺ cells supported early hematologic function allowing the animals to survive long enough for primitive progenitor cells to recover and sustain long-term hematopoiesis. The insertion of a genetic marker into the infused autologous cells or transplantation of allogeneic CD34⁺ cells will be required to prove the origin of cells responsible for hematopoiesis. Similarly, it cannot be determined whether partial hematologic recovery observed in one animal given marrow depleted of CD34⁺ cells was due to the recovery of stem cells in the irradiated recipient. It is also possible that inadequate depletion resulted in the infusion of a small number of CD34⁺ cells sufficient to allow the partial reconstitution of hematopoiesis. The development of more effective depletion methods and the use of genetic markers will be required to determine if either of these possibilities is correct.

A posttransplant syndrome of lymphadenopathy, atypical lymphocytosis, hemolytic anemia, and consumptive thrombocytopenia was observed in all transplanted animals surviving > 30 d. The illness has the characteristics of a viral disease,

but an etiology has not yet been identified. Animals receiving unmodified marrow, $CD34^+$ cells, or marrow depleted of $CD34^+$ cells have all developed the illness thus making it unlikely that the infusion of $CD34^+$ cells or the avidin-biotin immunoadsorption procedure per se caused this syndrome. Studies are in progress to identify the etiologic agent.

In humans and baboons, the $CD34^+$ marrow population is heterogeneous and contains immature B and T lymphocytes as well as myeloid progenitor cells (Andrews et al., manuscript submitted for publication; Andrews and Bernstein, unpublished observations; 3, 5, 19, 20). Furthermore, $CD34^+$ cells can be divided into functionally distinct progenitor populations based on expression of the CD33 antigen. In humans, the $CD33^-CD34^+$ progenitors are precursors of colony-forming cells in long-term marrow culture, while the $CD33^+CD34^+$ progenitors account for virtually all colony-forming cells (Andrews et al., manuscript submitted for publication). Studies to separate $CD34^+$ marrow cells into $CD33^-$, $CD33^+$, and lymphoid subpopulations will further define the nature and function of the progenitor cell populations required for reconstituting hematopoiesis in vivo.

The use of highly enriched populations of hematopoietic progenitor cells may have wide applicability to marrow transplantation. For example, if hematopoietic stem cells can be isolated without tumor cells, then this technique may provide an alternative approach to methods currently being used to deplete tumor cells ex vivo from the marrow of patients undergoing autologous marrow transplantation. Furthermore, positive selection could be used to isolate hematopoietic stem cells without T lymphocytes that cause graft-versus-host disease after allogeneic marrow transplantation. Finally, positive selection can provide a source of highly enriched hematopoietic progenitors for studies of gene transfer.

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