Human Erythroid Progenitor Cells Express Rhesus Antigens

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The expression of Rhesus antigens on hematopoietic progenitor cells was studied using monoclonal antibodies. Because these antibodies are not capable of lysing mature red blood cells in a complement-dependent cytotoxicity assay, fluorescence-activated cell sorting was performed. Using the monoclonal anti-Rh 29 antibody B10, 68% ± 6% of the mature erythroid progenitor cells (CFU-E) were sorted into the positive fraction, while only 2% ± 1% of the relatively immature erythroid progenitor cells (BFU-E), and 3% ± 1% of the granulocyte-macrophage progenitor cells (CFU-GM) were cultured from this same fraction. Thus up to a 15-fold enrichment of CFU-E could be obtained. In two experiments more than 4% of the cells in the positive fraction consisted of CFU-E; in one experiment even more than 7% did. Using fractionated cell sorting, the Rhesus antigens appeared to have a lower density on CFU-E than HLA-DR determinants. Antibodies against the Rhesus antigens can be applied to enrich erythroid-committed stem cells and to separate mature from immature erythroid progenitor cells.

THE EXPRESSION of red cell specific antigenic determinants on human hematopoietic progenitor cells (HPCs) has been studied by several authors.[1-8] In particular, antigens of the ABO blood groups have been studied extensively, since ABO incompatibility is relatively common in allogeneic bone marrow transplantation (BMT).[9] Antibodies in the recipient directed against the A or B antigens on the RBCs of the donor do not preclude prompt engraftment as measured by peripheral blood cell recovery and regeneration of HPCs after BMT,[9,10] suggesting that these antigens are probably not expressed on HPCs. In compliance with this, several authors, using human alloantisera or lectins, reported the absence of A and B antigens on committed myeloid or erythroid stem cells in vitro.[2,4] However, using monoclonal antibodies against A antigens and fluorescence-activated cell sorting (FACS), recent reports have suggested expression of A antigens on various HPCs.[5,6]

Similar to ABO blood group incompatibility, major incompatibility for Rhesus antigens between donor and recipient does not seem to affect engraftment after allogeneic BMT. As we previously showed, the presence of anti–Rhesus-C antibodies in a recipient after BMT did not inhibit in vivo the proliferation of both immature (BFU-E) and more mature (CFU-E) erythroid progenitor cells, as studied as early as 14 days after BMT.[10] In accordance with these observations, Rearden and Chiu[7] could not demonstrate the Rhesus-D antigen on CFU-E and BFU-E using anti-D alloantibodies in an indirect complement-dependent cytotoxicity assay.

However, their approach does not exclude the possibility of Rhesus antigens being expressed on HPCs because anti-D antibodies bound to mature RBCs do not fix complement. In this study, therefore, we have used monoclonal antibodies and FACS to investigate the expression of Rhesus antigens on HPCs. Evidence is presented that CFU-E do express Rhesus antigens, whereas BFU-E and CFU-GM do not. Therefore, these antibodies can be used for separating mature from more immature erythroid progenitor cells.

MATERIALS AND METHODS

Normal human bone marrow was obtained, after informed consent, by aspiration from the posterior iliac crests of donors for bone marrow transplantation. The bone marrow cells were collected in Hanks' balanced salt solution with 100 U/mL preservative-free heparin and diluted in RPMI 1640 with 5% fetal bovine serum (FBS; GIBCO, Grand Island, NY). The suspension was centrifuged over Ficoll-Isopaque (1.077 g/cm^3; 1,000 g for 20 minutes at 20 °C), and the interphase was harvested. The cells were then washed twice in RPMI + 5% FBS. Residual RBCs were lysed with an NH_4Cl buffer (0.155 mol/L NH_4Cl, 0.01 mol/L $KHCO_3$, 0.1 mmol/L EDTA, pH 7.4) for ten minutes at 0 °C. The cell suspension was washed three times in RPMI + 5% FBS (0 °C) and finally resuspended in RPMI + 20% FBS.

Cryopreservation. Mononuclear cells at a concentration of 20 × 10^6 cells per milliliter (before RBC lysis) were diluted 1:1 with a medium consisting of 40% RPMI, 40% FBS, and 20% dimethyl sulfoxide (DMSO) at 0 °C. Then, these cell suspensions were frozen in a computer-controlled freezer (Cryoson, Midden Beemster, the Netherlands) at a rate of 1 °C/min from 0 °C to −50 °C and then at a rate of 4 °C/min from −50 °C to −90 °C. The cells were stored in liquid nitrogen. Immediately before use the cells were thawed for one minute in a 37 °C water bath, diluted in HEPES-buffered RPMI + 20% FBS at 0 °C, and washed twice in the same medium at 0 °C. Because cell fragments might result in aggregation of cells in the cell sorter, the cells were centrifuged over Ficoll-Isopaque (1,000 g for 20 minutes at 20 °C). The interphase cells were then harvested, washed three times in RPMI + 5% FBS, and resuspended in RPMI + 20% FBS. Thus only 15% to 30% of the frozen cells were recovered in the interphase, resulting in a three- to fivefold enrichment for HPCs.

Antibodies. Monoclonal antibody B10 (anti-Rh 29, a common Rhesus antigen; IgG2a) has been described previously.[11] OKT3 (anti-T cell, IgG2a), used as a control antibody, was obtained from Ortho Pharmaceutical Corporation (Raritan, NJ). B8.11.2, a mono-

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clonal anti-HLA-DR backbone antibody (IgG2b), was a kind gift from Dr B. Malissen (Marseille, France). All antibodies were used as diluted ascites under saturating conditions (dilution 1:100). For indirect immunofluorescence, fluorescein isothiocyanate (FITC)-labeled goat antimouse Ig (Nordic Immunology, Tilburg, the Netherlands) was used.

Fluorescence-activated cell sorting. A quantity of 2×10^6 bone marrow cells in 0.2 mL was incubated with 0.2 mL monoclonal antiserum (standard dilution, 1:100) for one hour at 20 °C and washed three times in RPMI + 5% FBS. The cells were reincubated with FITC-labeled goat antimouse Ig for 30 minutes at 20 °C, washed twice, and resuspended to 3×10^6/mL in RPMI + 20% FBS. Using the FACS IV (Becton Dickinson, Sunnyvale, Calif; argon-ion laser beam tuned at 488 nm with an intensity of 300 mW), 2×10^5 cells were separated on the basis of fluorescence intensity into a negative fraction (channels 1 through 50, logarithmic scale) and one or two positive fractions (channels 51 through 255). The cutoff was chosen in channel 50 because using the fluorescent antibody only, the negative fraction ended approximately at channel 50. Dead cells and small lymphocytes were gated out after it had been demonstrated that these fractions did not contain HPCs (data not shown).[12] The cells were collected per fraction in RPMI + 50% FBS, centrifuged (600 g for ten minutes at 20 °C), and resuspended in Iscove's modified Dulbecco's medium (IMDM). Under identical conditions, 2×10^5 cells were run through the cell sorter without separation and cultured as a control ("unseparated control suspension").

Cell cultures. The culture medium consisted of 5% IMDM, 20% FBS (Rehatuin, Kankakee, Ill), 20% leukocyte-conditioned medium,[13] 5% 10^{-3} mol/L 2-mercaptoethanol, 5% deionized bovine serum albumin (Sigma Chemical Co, St Louis), 5% human transferrin, 1 U/mL (5%) erythropoietin (Connaught, step III, Toronto), and 35% methylcellulose 2.8%. Six replicates of 0.1 mL were plated into the wells of flat-bottomed microtiter plates (Flow Laboratories, McLean, Va) and cultured in a fully humidified atmosphere of 37 °C and 5% CO_2. CFU-E (a compact cluster of eight to 64 hemoglobinized cells, rapidly dissipating after day 8) was scored on day 7. CFU-GMs (granulocytic, monocytic, or eosinophilic aggregates of more than 20 cells) were counted on day 10, and the number of BFU-Es was scored on day 14.

RESULTS

Using the monoclonal anti-Rh 29 antibody B10, 68% ± 6% (mean ± SE) of the CFU-Es were recovered from the positive fraction after cell sorting, expressed as a percentage of the number of CFU-Es, recovered from the unseparated control suspension (Table 1). Using the control monoclonal antibody OKT3, only 3% ± 1% of the CFU-Es were sorted into the positive fraction (difference significant, $P < .0001$, Wilcoxon test).

As shown in Table 2, up to a 15-fold enrichment of CFU-E (8.4 ± 1.4, mean ± SE) could be obtained in the Rhesus positive fractions as compared with the unseparated control suspension. When cryopreserved cells were used (experiments 7 through 12), there was only a 5.5 ± 1.5 (mean ± SE) enrichment of CFU-E because more non-erythroid cells were nonspecifically sorted into the positive fraction, whereas the enrichment factor of the CFU-Es from the fresh suspensions was 11.2 ± 1.9 (experiments 1 through 6). In two experiments more than 4% of the cells in the Rhesus positive fraction consisted of CFU-E. In experiment 12, even 7.3% of the cells appeared to be CFU-E. Both after incubation with anti-Rhesus antibodies and after incubation with OKT3 antibodies almost all BFU-Es and CFU-GMs were recovered from the negative fractions (no significant differences), as shown in Table 3. Using anti-HLA-DR antibodies, approximately 90% of all HPCs were sorted into the positive fractions.

As demonstrated in Table 3, there appeared to be a difference in the percentages CFU-Es sorted into the negative or positive fractions after incubation with anti-Rhesus antibodies as compared with anti-HLA-DR antibodies (differences significant, $P < .01$ and $P < .02$, respectively, Wilcoxon test). To study further the differences in density of the Rhesus and HLA-DR antigens, both determinants were analyzed within one experiment using the same bone marrow cell suspension and fractionated cell sorting. As shown in Fig

Table 1. Recovery of CFU-E After FACS Using Monoclonal Anti-Rhesus Antibody B10

Experiment No.	Negative Fraction			Positive Fraction			Unseparated Control
	Cells (%)	CFU-E		Cells (%)	CFU-E		
		No.*	%†		No.*	%†	No. CFU-E*
1	96	79	(53)	4	87	(59)	148
2	95	134	(20)	5	418	(62)	676
3	94	97	(17)	6	465	(80)	583
4	98	668	(137)	2	115	(24)	487
5	79	35	(15)	21	183	(76)	241
6	91	20	(16)	9	103	(82)	126
7	78	106	(24)	22	439	(99)	442
8	81	433	(38)	19	943	(84)	1,128
9	86	195	(24)	14	648	(79)	824
10	80	148	(24)	20	345	(56)	616
11	89	283	(33)	11	352	(41)	849
12	94	127	(11)	6	881	(73)	1,208
Total	88 ± 2	194 ± 56	(34 ± 10)	12 ± 2	415 ± 87	(68 ± 6)	611 ± 106
OKT3	79 ± 4	553 ± 97	(87 ± 5)	21 ± 4	19 ± 8	(3 ± 1)	662 ± 114

Cryopreserved cells were used in experiments 7 through 12. Totals are the means ± SE.

*Total number of CFU-E per fraction.

†CFU-E per fraction as percentage of unseparated control.

Table 2. Enrichment of CFU-E Using Anti-Rhesus Antibodies and FACS

Experiment No.	Number of CFU-E per 10^5 Sorted Cells			Enrichment Factor*	
	Negative Fraction	Positive Fraction	Unseparated Control	Negative Fraction	Positive Fraction
1	41	1,088	74	0.6	14.7
2	70	4,644	338	0.2	13.7
3	51	4,227	292	0.2	14.5
4	341	2,875	244	1.4	11.8
5	22	436	121	0.2	3.6
6	11	572	63	0.2	9.1
7	68	976	221	0.3	4.4
8	266	2,549	564	0.5	4.5
9	113	2,314	412	0.3	5.6
10	93	841	308	0.3	2.7
11	159	1,600	425	0.4	3.8
12	68	7,342	604	0.1	12.2
Total	109 ± 30	2,455 ± 623	306 ± 53	0.4 ± 0.1	8.4 ± 1.4

Cryopreserved cells were used in experiments 7 through 12. Totals are the means ± SE.
*Enrichment factor compared with unseparated control.

1, 83% of the sorted CFU-Es were recovered from the weakly positive fraction (fluorescence channels 51 through 120), when incubated with the anti-Rhesus antibody, and no CFU-Es were recovered from the strongly positive fraction (channels 121 through 255). Using anti-HLA-DR antibodies, 42% of the CFU-Es were recovered from the strongly positive fraction, indicating that, in fact, on CFU-E, the expression of HLA-DR antigens was higher than that of Rhesus antigens.

DISCUSSION

Analysis of the surface antigenic determinants can be used to characterize HPCs during their differentiation to mature hematopoietic cells. Some antigens, like HLA-DR, are present on all HPCs,[14] but their expression may decrease with maturation. Other determinants, like the granulocyte antigen reacting with monoclonal antibody 1G10, are only weakly expressed on immature precursor cells but increase in density during maturation.[15] Whereas some antigens, like HLA-DR, are expressed on HPCs of all lineages,[14] other determinants, like EP-1, appear to be lineage-specific.[8] Determinants of the ABO system could be demonstrated on human erythroid-committed hematopoietic stem cells using monoclonal antibodies,[5,6] despite the inability of several investigators to detect these antigens on HPCs using alloantisera.[2,4]

Until now, no evidence of the expression of Rhesus antigens on HPCs has been published. Although Rhesus antigens could be demonstrated on the most immature, erythroid precursor cells recognizable in light microscopy, ie, the pro-erythroblasts, these antigens could not be detected on CFU-E.[7] However, alloantibodies against Rhesus antigens are not capable of complement fixation. Therefore, Rearden and Chiu[7] used an indirect cytotoxicity assay with anti-Ig antibodies in an attempt to eliminate HPCs after incubation with anti-D, but no inhibition of HPCs was found.

As shown in Table 1, the Rhesus "backbone" antigen (Rh 29) as defined by monoclonal antibody B10 appears to be present on CFU-E. Whereas in 12 experiments using OKT3 as control antibody, only 3% ± 1% (mean ± SE) of the CFU-Es were sorted into the positive fraction, 68% ± 6% of the CFU-Es were cultured from the positive fraction after incubation with the anti-Rhesus antibody. Thus up to a

Table 3. Recovery of HPC After FACS Using Anti-Rhesus, Anti-HLA-DR, and OKT3 Antibodies

Fraction	Percentage of Unseparated Control		
	Anti-Rhesus (n = 12)	Anti-HLA-DR (n = 8)	OKT3 (n = 12)
CFU-E			
Negative	34 ± 10	11 ± 4	87 ± 5
Positive	68 ± 6	89 ± 5	3 ± 1
BFU-E			
Negative	91 ± 5	6 ± 3	91 ± 5
Positive	2 ± 1	93 ± 6	3 ± 2
CFU-GM			
Negative	85 ± 5	7 ± 3	91 ± 7
Positive	3 ± 1	93 ± 8	6 ± 3

Results shown are the means ± SE.

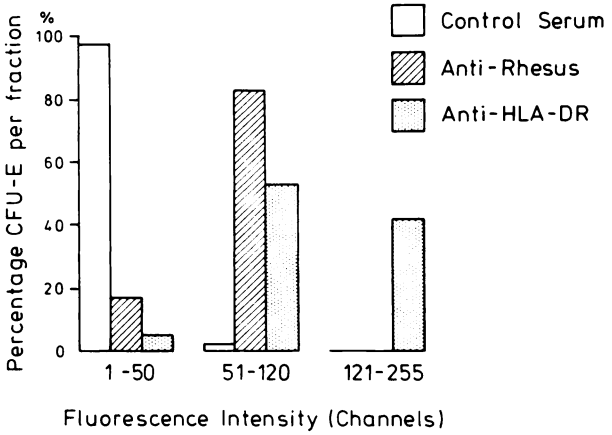

Fig 1. FACS using anti-Rhesus and anti-HLA-DR monoclonal antibodies. Recovery of CFU-Es per fraction. Channels 1 through 50 indicate negative fluorescence; channels 51 through 120, weakly positive fluorescence; channels 121 through 255, strongly positive fluorescence.

15-fold enrichment of CFU-E could be achieved, resulting in a maximally 7.3% concentration of CFU-E in the positive fraction (Table 2). No immature erythroid-committed HPCs, ie, BFU-Es, or CFU-GMs could be cultured from the same Rhesus positive fraction (Table 3). These data show that using anti-Rhesus antibodies and FACS, CFU-E can be separated from its precursor cell, the BFU-E. We previously demonstrated that, although CFU-E expresses HLA-DR determinants,[10] the expression of these Class 2 determinants on CFU-E is relatively low as compared with their density on CFU-GM and BFU-E.[16] As shown in Fig 1, even fewer anti-Rhesus antibodies than anti-HLA-DR antibodies are bound to the CFU-E, indicating that the expression of Rhesus antigens on CFU-E is weak. This weak expression does not necessarily imply that, after BMT over a major Rhesus barrier, severe complications are to be expected in a recipient with antibodies directed against these blood groups of the donor. Engraftment can be expected to be normal, since these antigens are not expressed on BFU-E and CFU-GM and therefore presumably neither on the pluripotential hematopoietic stem cells. Comparable to ABO incompatibility, only in cases of high antibody titers in the recipient, delayed erythroid recovery is to be expected.[9,10]

We have demonstrated differential expression of Rhesus antigens on erythroid progenitor cells. Although the expression of Rhesus antigens on CFU-E probably does not have clinical implications, antibodies against these antigens can be used to separate the immature (BFU-E) from the more mature (CFU-E) erythroid-committed stem cells.

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