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Separation of Hemopoietic Cells From Adult Mouse Marrow by Use of Monoclonal Antibodies

By Trang Hoang, David Gilmore, Don Metcalf, Steve Cobbold, Suzanne Watt, Mike Clark, Mark Furth, and Herman Waldmann

Primitive hemopoietic progenitor cells from adult mouse marrow have been substantially enriched by virtue of a negative selection procedure with monoclonal antibodies. It has also been possible to segregate erythroid progenitor cells at distinct stages of differentiation on the basis of their cell surface antigens. This has been achieved with two monoclonal antibodies reactive with the mature elements of bone marrow. YBM 34.3 binds to a heat-stable antigen expressed on B lymphocytes, neutrophils, and cells of the erythroid lineage. YBM 6.1 reacts with cells of the neutrophil, eosinophil, and monocyte series but does not bind to colony-forming cells. Separation is achieved by indirect immunoadsorption (panning) with YBM 34.3 on Protein-A-coated plastic plates followed by FACS II cell sorting with YBM 6.1. The combined procedures yield a marrow population containing 58% immature cells (blasts, promyelocytes, and myelocytes) and 9.5% clonogenic cells. In addition, differential binding of YBM 34.3 can be used to segregate erythroid progenitor cells at distinct stages of differentiation (day 7 BFU-E, day 5 BFU-E and CFU-E) either by cell sorting or panning. It is shown that both techniques give a comparable degree of resolution of the different cell types with, however, an appreciable advantage of panning over cell sorting in allowing the rapid handling of large numbers of cells.

HEMOPOIETIC progenitor cells require specific glycoprotein stimulators for their proliferation and differentiation in vitro. Progress in understanding how these molecules operate at the cellular and molecular level depends on the availability of purified factors on the one hand and purified target cells on the other. To date, three such stimulatory factors with restricted lineage specificity have been purified to homogeneity.[1-3] These have been shown to differ from factor(s) that act on multipotential cells and their early committed progeny.[4] Although characterization of stimulatory factors is relatively well documented, purification of target cells has been hindered by the cellular heterogeneity of the hemopoietic tissue and the lack of specific labels. At present, these cells can be detected only by their capacity to form colonies in vivo (CFU-S) or in vitro (CFU-C or CFC). With such assay systems, colony-forming cells are found to constitute only a small fraction of all cells in adult marrow (0.1%–0.4%). Furthermore, they are heterogeneous and vary in their capacity for proliferation and differentiation along one or more pathways. For example, in the erythroid pathway, it has been shown that precursors giving rise to day 2 colonies (CFU-E) have a more limited proliferative potential than those giving rise to day 7 colonies (BFU-E). In addition, while all the mature precursors (day 3 BFU-E and CFU-E) are erythroid committed, a large proportion of the more primitive precursors (day 7 BFU-E) give rise to erythroid colonies that also contain megakaryocytes.[5]

Therefore, much effort has gone into finding techniques for enrichment and segregation of progenitor cells based on physical parameters[6-11] and cell surface markers.[12-14] The approach can either be based on positive[15] or negative selection procedures.[16] With a combination of all these approaches, Nicola et al. were able to achieve a 36–50-fold enrichment of progenitors in fetal liver. The methods used were gravity sedimentation followed by dual fluorescence cell sorting with an antineutrophil serum and a fluorescent lectin.[12] The recent advent of monoclonal antibody technology has encouraged the search for reagents that can become standard laboratory tools for enrichment procedures and for segregation of progenitors at different stages of development. This article describes the use of two such reagents for these purposes. One monoclonal antibody, YBM 34.3, has a broad reactivity binding most end-stage cells in adult mouse marrow (red cells, B lymphocytes, some neutrophils, and monocytes). The second monoclonal antibody, YBM 6.1, detects an antigen expressed on all neutrophils, eosinophils, and monocytes. Immunoadsorption with the former leaves a population enriched for progenitors. Residual myeloid cells are removed by FACS II cell sorting with the second reagent.

On the basis of differential binding of the monoclonal antibody YBM 34.3, it has also been possible to

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show that different stages of erythroid differentiation (day 7 BFU-E, day 5 BFU-E and CFU-E) vary in their expression of the relevant surface determinant. They can therefore be separated for studies of erythropoiesis at the molecular level.

MATERIALS AND METHODS

Monoclonal Antibodies

The monoclonal antibodies YBM 6.1, YBM 34.3, and YBM 29.2 were derived from a fusion between the rate myeloma line Y3/Ag 1.2.3 and spleen cells from DA rats immunized with CBA/Ca mouse bone marrow cells by the method of Galfre and Milstein.[17] The two antibodies used for cell separation, YBM 6.1 and YBM 34.3, were selected by virtue of their reactivity pattern, after recloning, in binding assays on panels of normal and tumor cell lines of the hemopoietic system. Both antibodies are of the IgG class and only one, YBM 34.3, binds Protein A.

The hybridomas were grown in Iscove's modified Dulbecco's medium (IMDM, GIBCO, Grand Island, N.Y.) supplemented with 1% fetal calf serum (FCS, Sera Lab, Crawley Down, England). The culture supernatants were collected 24 hr after the cells reached confluency. They were precipitated at 50% saturation of ammonium sulphate, redissolved in phosphate-buffered saline (PBS, pH 7.4), aliquoted, and stored at −20°C in presence of 0.1% bovine serum albumin (BSA, Sigma, St. Louis, Mo.). The protein concentration was determined by absorption at 280 nm prior to the addition of BSA.

Two monoclonal antibodies were used for estimating T and B lymphocytes by fluorescence microscopy: YBM 29.2, a rat anti-mouse Thy-1[18] (Cobbold and Waldmann, unpublished) and SM1/45HL, a rat anti-mouser immunoglobulin raised against MOPC 104E and specific for mouse μ chains.[19] YBM 29.2 antibody was collected as ascitic fluid, precipitated with ammonium sulphate as above, and dialyzed against 0.1 *M* $NaHCO_3/Na_2CO_3$ buffer, pH 9.3, for coupling. YBM 29.2 was coupled with FITC (fluorescein isothiocyanate, Sigma, dissolved at 1 mg/ml in DMSO) at a ratio of 25 μg/mg of immunoglobulin for 2 hr at room temperature with constant rotation. The coupled antibody was dialyzed against PBS at 4°C and stored in aliquots at −20°C in presence of 0.1% NaN_3 and 1% BSA. The SM1/45HL was purified from serum-free tissue culture supernatant by precipitation with ammonium sulphate and was coupled to biotin for subsequent detection with rhodamine-avidin.[20] Biotin was added (Biotinyl-N-hydroxy-succinimide ester, BA106, E-Y Laboratories, U.S.A.) at a concentration of 120 μg/mg of immunoglobulin for 2 hr at room temperature. The reagent was then dialyzed and stored as above.

Cell Preparation

CBA/Ca mice (male, 6–8 wk old) were used throughout the experiment. Bone marrow cells were collected from the femurs in bicarbonate-free IMDM supplemented with 5% heat-inactivated FCS. Sodium chloride added at a concentration of 35 m*M* substituted for sodium bicarbonate in this medium, which was used routinely for handling cells and for panning. Erythrocytes were lysed by treating the cell suspension with 0.168*M* NH_4Cl for 10 min at room temperature. Nucleated cells were washed twice and resuspended in a minimum volume of HEPES-buffered Eagle's medium without phenol red (Flow, Detroit, Mich.) supplemented with 1% BSA and 0.02% sodium azide for labeling with the antibodies.

Cell Separation by "Panning"

The preparative immunoabsorption step was carried out essentially as described by Nash,[21] with minor modifications.

Preparation of Protein-A-coated plates. Tissue culture plates (Falcon 3001, 35mm, Oxnard, Calif.) were coated with Protein A as follows: 1 ml of human IgG (fraction II, Sigma, 500 μg/ml in saline) was added to each plate with an equal volume of carbodiimide [1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl-carbodiimide methotoluene sulfonate, Sigma, 1 mg/ml in saline] for 1 hr at room temperature. The plates were washed twice, and blocked with 2 ml IMDM containing 5% heat-inactivated FCS for 10 min. Protein A (Pharmacia, Piscataway, N.J., 50 μg/ml in PBS/0.2% NaN_3) was added at a final volume of 2 ml/plate for 1 hr at room temperature. The plates were washed twice with PBS and stored in PBS/0.2% NaN_3 at 4°C for several weeks without decline in binding capacity. The Protein A solution was kept at −20°C and recycled for at least 9 experiments without loss of activity.

Cell separation. Bone marrow cells (5×10^6) were labeled with various concentrations of the monoclonal antibody YBM 34.3 for 30 min at ice temperature. The suspension was diluted fivefold and spun through a 1-ml layer of heat-inactivated FCS. The cell pellet was carefully resuspended in 2 ml of medium and pipetted onto Protein-A-coated plates for panning. The cells were left to sediment for 30 min at 4°C, the dish was centrifuged (1 min, 300 *g*) and incubated for another 15 min. The plates were then filled with 8 ml of medium, and the lid replaced carefully to avoid air bubbles. They were inverted for 1 hr in the cold for removal of nonadherent cells. In the control group, unlabeled cells were processed and panned under the same conditions. Viable cells recovered after panning were counted by Trypan blue exclusion. Cells recovered in the antibody-treated groups were expressed as percent of cells recovered in the control group.

Cell Sorting With the FACS II

FACS analysis and cell sorting was performed by a two-step indirect labeling of the cells. In the first step, cells ($2–5 \times 10^7$/ml) were labeled with YBM 34.3 or YBM 6.1 for 30 min and washed through a layer of heat-inactivated FCS as above. The second step involved an incubation with either FITC-Protein A (Pharmacia, 20 μg/ml final concentration) or a FITC-rabbit $F(ab)_2$ anti-rat Ig in a total volume of 200–400 μl. After 30 min, the cells were washed through a layer of heat-inactivated FCS and resuspended in Eagle's medium without phenol red containing 1% BSA and 0.02% NaN_3 at a final cell concentration of 5×10^6/ml for sorting. As control, cells were incubated with the second reagent alone. The rabbit anti-rat IgG had been absorbed with mouse spleen cells and purified by affinity chromatography on a column of rat IgG. The $F(ab)_2$ fragment was prepared by pepsin digestion, then labeled with FITC as above.

Light scattering properties and fluorescence of cells were determined by use of a FACS II (Becton Dickinson, Sunnyvale, Calif.) as described by Visser et al.[10] The population with low forward light scattering properties (channels 75–120) is referred to as small cells. These consist of small lymphocytes and nucleated red cells. The population referred to as large cells (channels 120–250) has relatively high forward light scattering properties and include blast cells, all myeloid cells in the bone marrow, medium to large sized lymphocytes and erythroblasts.

Two-Color Immunofluorescence Microscopy for Enumeration of Lymphocytes

After panning or cell sorting, bone marrow cells (50,000/100 μl total volume) were incubated with biotin-coupled SM1/45HL (200 μg/ml) and FITC-coupled YBM 29.2 (20 μg/ml) for 30 min at 4°C in 1-ml microfuge tubes in PBS or a biotin-free medium containing 1% BSA and 0.02% sodium azide. The cells were washed, resuspended in 200 μl of PBS for staining with rhodamine avidin (Sigma,

20 μg/ml, 10 min), diluted in a biotin-containing medium (IMDM) to stop the reaction, washed, resuspended in 20 μl of PBS, and assessed for red and green fluorescence (×60, oil immersion lens). Propidium iodide (Calbiochem-Behring Co., La Jolla, Calif.) was added in the final wash at a concentration of 50 μg/ml. Dead cells were stained strongly red.[22] Where indicated, biotin-coupled YBM 29.2 and rhodamine-avidin was used instead of the FITC-coupled reagent.

Culture Technique

Bone marrow cells were plated (Falcon 1008, 35 mm) at concentrations ranging from 5000 to 50,000/ml in IMDM containing 0.9% methylcellulose (w/v, 4000 cps, Fluka, Buchs SG, Switzerland), 10% heat inactivated FCS (v/v, Sera Lab), delipidated and deionized bovine serum albumin (Hoechst, Frankfurt, W. Germany), iron-saturated human transferrin (Hoechst), L-α-dipalmitoyl phosphatidyl choline (Sigma), oleic acid (Sigma), and cholesterol (Sigma) as described by Iscove et al.[23] The cells were stimulated with conditioned medium from a WEHI-3 subclone, kindly donated by Dr. M. Dexter, at an optimum concentration of 10% (v/v). Erythropoietin was added at 1 U/ml.

Erythroid colonies were scored on days 2, 3, 5, and 7 of culture essentially as described by Gregory.[24] However, all erythroid colonies on day 3 were classified together, regardless of their growth pattern, as single clusters or bursts consisting of several subclusters. Nonerythroid colonies that contained 3 or more cells on day 2 were classified as clusters, those containing 50 or more cells on day 7 were called colonies. The latter were further classified as granulocyte-macrophage (GM) if they contained either granulocytes, macrophages, or both. Colonies containing 3 or more cell types (including erythroid) were defined as multipotential (M/E).

RESULTS

Reactivity of YBM 34.3 and YBM 6.1 With Normal Hemopoietic Cells

FACS II analysis of bone marrow cells labeled with YBM 34.3 showed that 83% of the cells were labeled (Table 1). The reactivity was comparable to that of the M1/69.16 monoclonal antibody described by Springer,[25] which is directed against a heat-stable antigen present on red cells, granulocytes, monocytes, B lymphocytes, thymocytes but not peripheral T lymphocytes. Cell sorting with YBM 34.3 indicated that the antibody bound to nucleated red cells, lymphocytes, neutrophils, some monocytes, and very few blast cells (Table 1A). Immunofluorescence staining of the positive and negative cells with a biotinylated YBM 29.2 and rhodamine-avidin showed that Thy-1-positive T cells are negative for YBM 34.3 (Table 1B). Negative cells were further sorted into small cells and large cells. Almost all Thy-1-positive cells were recovered in the small cell fraction.

In the experiment shown in Fig. 1, bone marrow cells labeled with YBM 34.3 were sorted into three fractions according to their fluorescence intensity. The different types of colony-forming cells in each fraction was determined as described (Fig. 1 C and D). The fraction with highest fluorescence intensity contained almost all CFU-E, while day 7 BFU-E and multipotential colony-forming cells (BFU-M/E) were mainly in the negative fraction. Day 5 erythroid colonies deriving from cells at intermediate stages of differentiation between day 7 BFU-E and CFU-E were scored separately as pure or mixed clones. The pure erythroid clones peaked in the fraction with intermediate fluorescence intensity, while the mixed ones were mainly in the negative fraction. The results indicated that expression of the antigen detected by YBM 34.3 increases with differentiation along the erythroid pathway.

Nonerythroid colonies were scored on day 2 and day

Table 1. Separation of Mouse Bone Marrow Cells With YBM 34.3 and the FACS II

	Starting Bone Marrow	Cell Fraction			
		Small Cells	Large Cells	Small Cells	Large Cells
(A) Cell size					
Fluorescence	—	Negative	Negative	Positive	Positive
Percent total cells	—	3.9	13.2	26.4	56.4
Percent cell type					
Blasts	4	n.d.	22	0	1
Promyelocytes myelocytes	6		9	0	8
Metamyelocytes neutrophils	48		3	0	48
Eosinophils	0		1	0	4
Monocytes	7		31	0	7
Lymphocytes	16		32	93	21
Nucleated red cells	19		2	7	11
(B) Percent Thy-1-positive cells in each fraction	3	32	2	0	2

(A) Frequency and distribution of the different cell types.

(B) Staining of the different FACS-sorted fractions with YBM 29.2 (anti-Thy-1). Nucleated bone marrow cells were labeled with YBM 34.3 (30 μl/ml) and FITC-Protein-A. Prior to sorting, cell aggregates were removed by sedimentation through a layer of FCS. Cells were sorted according to their fluorescence intensities: negative, channels 0–18; positive, channels 20–250. The FITC-Protein-A control analyzed under the same conditions had 2.5% positive cells. Small cells: channels 75–120 (forward light scatter). Large cells: channels 120–250 (forward light scatter).

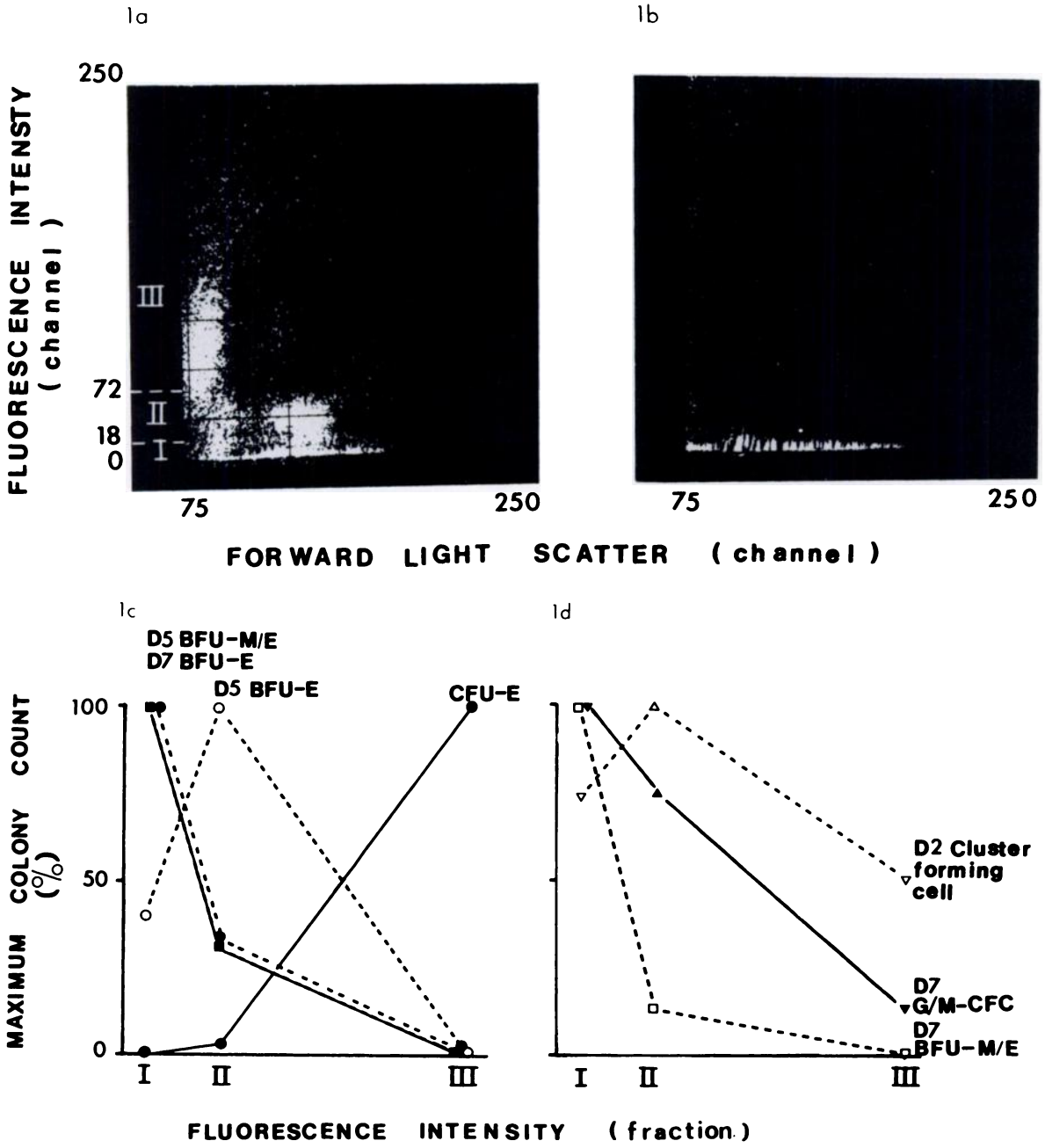

Fig. 1. FACS II analysis of bone marrow cells labeled with YBM 34.3. Nucleated bone marrow cells were labeled with YBM 34.3 (30 μg/ml) and FITC-Protein-A (100 μg/ml) (a) or FITC-Protein-A alone (b). Cells were sorted into three fractions according to their fluorescent intensities. Fraction I (channels 0–18) contained 26.7% of the cells, fraction II (channels 20–72) 38.8%, fraction III (channels 73–248) 34.5%. The distribution of the different types of colony-forming cells in the sorted fractions was normalized to 100% (c and d). Maximum colony count per 10^5 cells in the corresponding fraction was the following: CFU-E, 540; day 3 BFU-E, 300; day 5 BFU-E, 50; day 5 BFU-M/E, 160; day 7 BFU-E, 250; day 7 BFU-M/E, 460; cluster-forming cells, 1980; day 7 GM CFC, 670.

7. The frequency of proliferating cells counted on day 2 was maximum in the fraction with intermediate fluorescence intensity, while the peak of day 7 GM CFC corresponds to the negative fraction. The corresponding results for YBM 6.1 are shown in Fig. 2 and Table 2.

Panning of Bone Marrow Cells With YBM 34.3

Mouse bone marrow cells (5×10^6) depleted of red cells were labeled with increasing concentrations of YBM 34.3 (Table 3). At high concentrations, the antibody tended to agglutinate cells. Large clumps

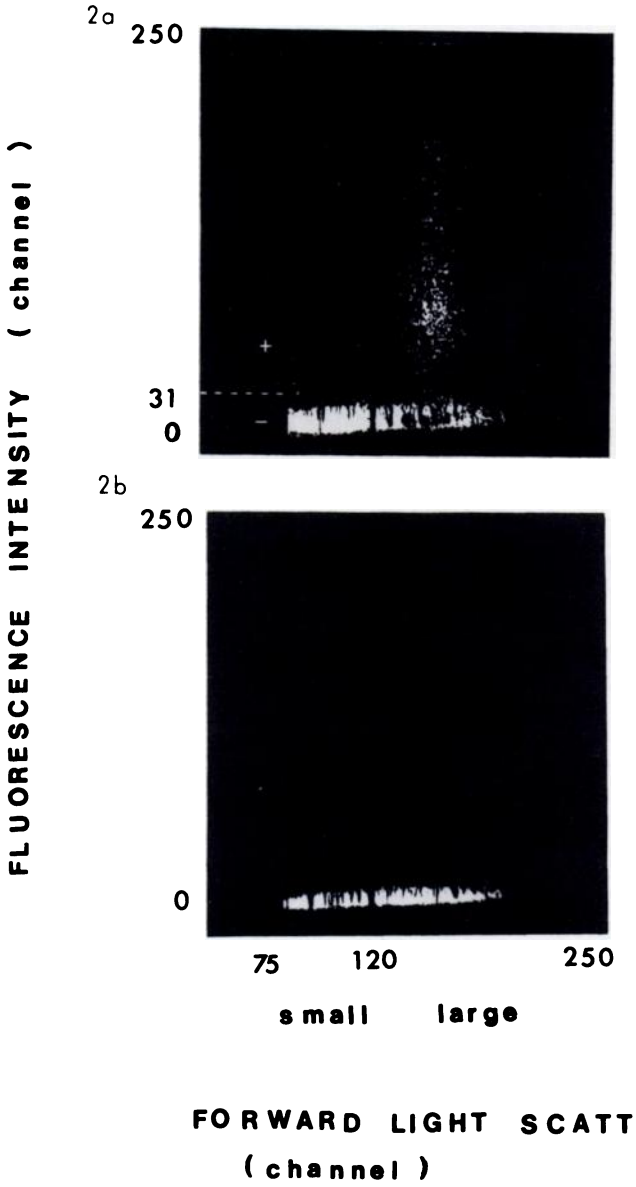

Fig. 2. FACS II histograms of bone marrow cells labeled with YBM 6.1 and FITC-F(ab)$_2$ rabbit anti-rat Ig (a) or FITC-F(ab)$_2$ alone (b, control), as indicated in Table 2.

were therefore removed prior to panning by sedimentation at 1*g* through a layer of heat-inactivated FCS as described by Shortman.[26] Small agglutinates were removed subsequently by the panning procedure.

Panning was performed as described in Materials and Methods. The distribution of the different types of colony-forming cells in the nonadherent fractions was determined. In the control, where cells were panned on Protein-A-coated plates but without using antibody, 30% of the cells were lost, although virtually no colony-forming cells were removed. This implies that the loss was nonrandom. These figures refer to the panning step itself, the overall process, including washes, etc., resulted in a 66% loss of all cells and 40%–60% of colony-forming cells. This presumably means that half the cells were lost during the washing and labeling processes and that these losses were random. Similar figures will apply to cell sorting, since the labeling steps are identical to those for panning.

In Table 3 the results for panning with YBM 34.3 are compared with those for the control, in that the cell recovery from the latter has been normalized to 100%. The three different concentrations of the antibody removed 88% or 89% of the cells and enriched day 7 colony-forming cells by a factor of 5–7 compared with the control (with respect to the starting population, the enrichment was more like a factor of 7 or 9, for the reasons given above).

This means that some loss of day 7 CFC occurred (approximately 30%–40%), otherwise they would have

Table 2. Separation of Mouse Bone Marrow Cells With YBM 6.1 and the FACS II

Cell Size	Starting Bone Marrow	Cell Fraction		
		Small Cells	Large Cells	Total Cells
Fluorescence	—	Negative	Negative	Positive
Percent total cells	—	51	24	25
Percent cell type in each fraction				
Blasts	4	0	17	1
Promyelocytes myelocytes	4	0	3	7
Metamyelocytes neutrophils	32	0	2	75
Eosinophils	1	0	0	4
Monocytes	11	0	0	9
Lymphocytes	22	87	51	5
Nucleated red cells	26	13	27	0

Nucleated bone marrow cells were labeled with YBM 6.1 (100 μg/ml) and FITC-F(ab)$_2$ rabbit anti-rat Ig. Cells were sorted according to their fluorescence intensities: negative, channels 0–30; positive, channels 32–250. Small and large cells were separated as in Table 1.

been enriched by a factor of about 9 (100/11) with respect to the control. The question therefore arises whether that 30%–40% of day 7 CFC were removed randomly or specifically, i.e., do they differ in some way, particularly with regard to the antigen detected by YBM 34.3, from the 60%–70% of the day 7 colony-forming cells recovered from the panning procedure? There is no direct answer to this question, although comparison with the results obtained by FACS II cell sorting with the same antibody suggests that a minor proportion of day 7 colony-forming cells is labeled. These would therefore be removed in the panning experiment.

A similar question arises with regard to day 2 CFU-E and day 3 BFU-E. Table 3B shows that for YBM 34.3 at 7 μg/ml, day 2 CFU-E were present in the same proportions in the panned sample as in the control. On the face of it, this means that CFU-E were neither enriched nor depleted. However, since the panned sample contained only 12% of the number of cells in the control, it means in fact that 88% of the cells and 88% of the CFU-E have been removed. As the YBM 34.3 concentration increases, the proportion of CFU-E in the panned sample decreases, so that at 30 μg/ml, it is down by a factor of 8 compared with the control. This of course implies that more than 98% of the CFU-E have been removed. At the same antibody concentration, day 3 BFU-E showed a sevenfold decrease in frequency compared with the control, implying that again more than 98% were removed.

Table 3. Panning of Mouse Bone Marrow Cells With YBM 34.3

	Starting Bone Marrow	Control	Monoclonal Antibody YBM 34.3 (μg/ml)		
			7	15	30
Cell recovery	—	100	12	12	11
(A) Morphology (%)					
Blast	3	6	17	15	15
Promyelocytes myelocytes	5	9	14	11	17
Metamyelocytes neutrophils	37	28	11	12	14
Eosinophils	1	2	0	3	2
Monocytes	2	3	15	23	13
Lymphocytes	24	24	35	31	33
Nucleated red cells	28	28	8	5	6
(B) Colony-forming cells (per 10^5)					
Day 2 CFU-E	769	864	876	144	108
Day 3 BFU-E	ND	704	540	270	108
Day 5					
BFU-E	22	24	169	79	83
BFU-M/E	11	24	146	146	138
Day 7					
G/M-CFC	116	154	1,011	763	780
BFU-E	26	49	247	180	211
BFU-M/E	24	57	337	281	404

(A) Frequency and distribution of the different cell types before and after panning.

(B) Colony-forming cell content of the nonadherent fractions. Results expressed as number of colonies per 10^5 in each fraction. The cells were plated at concentrations equivalent to 5×10^4 cells in the control group.

Day 5 colonies did not show such a dramatic decrease but exhibited an interesting difference between pure and mixed erythroid colonies. The pure erythroid ones increased in frequency compared with the control by a factor 3 or 4, implying that 62% of them were removed by panning. However, the mixed erythroid ones increased by a factor of 5–6, implying that 46% were removed. The latter results are very similar to those for day 7 erythroid colonies.

These effects on erythroid progenitors correlated with the morphological data (Table 3A) where nucleated red cells decreased in frequency by a factor of about 5, corresponding to an almost 98% loss. The nucleated red cells that remained in the panned population were primitive erythroblasts with very basophilic cytoplasm as compared to those in the control, which included all the differentiated stages of the erythroid series.

The above results for panning compared well with those obtained by FACS II cell sorting with the same antibody. This can be seen from Fig. 1 (C and D) if one imagines a vertical line displaced from the y-axis as indicative of the threshold above which cells would be removed by panning. This threshold would be expected to move to the left as the antibody concentration used in panning was increased. In particular, the cell sorting results would predict that day 5 pure erythroid colonies would be depleted before the mixed ones, as well as of course the almost complete removal of day 2 CFU-E.

With regard to the question concerning specificity of removal, the results for lymphocytes are of some interest. Table 3A shows that the proportion of lymphocytes in the panned sample was slightly greater than in the control (by a factor of 1.3–1.6). Again, due to the fact that the panned sample contained only 11% or 12% of the number of cells in the control, this corresponds to a loss of 85% of the original lymphocytes compared to the control. In this instance, there is evidence that the depletion was selective in that the lymphocytes in the panned sample contained no cells bearing surface IgM and were in fact enriched for Thy-1-positive cells (data not shown).

Two-Step Separation of Bone Marrow Cells by Panning With YBM 34.3 and Cell Sorting With YBM 6.1

Mouse bone marrow cells were panned with YBM 34.3 at a concentration of 30 μg/ml (step 1). Nonadherent cells were collected and labeled with YBM 6.1 (100 μg/ml) and a fluorescein-tagged $F(ab)_2$ rabbit anti-rat IgG for sorting (step II). Positive and negative cells in the large cell population were collected. Identification of the cells in each fraction is shown in Table 4.

As discussed in the previous section, the panning step removed large numbers of nucleated red cells, lymphocytes, and neutrophils. Myeloid cells that had not reacted with YBM 34.3 were removed by labeling with YBM 6.1 and sorting on a FACS II cell sorter in the following way. Cells with low forward light scatter

Table 4. Two-Step Separation of Adult Mouse Bone Marrow Cells by Panning With YBM 34.3 and FACS II Cell Sorting With YBM 6.1

	Starting Bone Marrow	Step I Panning With YBM 34.3	Step II Cell Sorting With YBM 6.1	
Cell fraction	—	Nonadherent cells	Large negative	Large positive
Percent starting population	—	10	4.8	3.6
(A) Morphology				
Blasts	4	15	47	3
Promyelocytes myelocytes	3	10	11	8
Metamyelocytes neutrophils	27	28	1	69
Eosinophils	0	2	0	0
Monocytes	4	10	2	16
Lymphocytes	34	27	35	4
Nucleated red cells	28	8	4	0
(B) Colony-forming cells (per 10^5)				
Day 2				
CFU-E	756	ND	500	0
Cluster-forming cells	1,356		9,000	6
Day 7				
GM	119	ND	3,691	116
E	25		200	0
M/E	32		575	17

(A) Frequency and distribution of the different cell types.

(B) Colony-forming cell content of the FACS-sorted fractions. Results expressed as number of colonies per 10^5 cells. Cells from the enriched fraction were cultured at a concentration of 5000/ml. Cells were sorted on the basis of their fluorescence intensities and forward light scatter properties, as in Table 2.

properties were gated out, this excluded all Thy-1-positive cells. The remaining cells with medium to high forward light scatter were divided into two fractions, one positive for YBM 6.1, the other negative. The negative fraction, i.e., medium to large cells negative for both YBM 34.3 and YBM 6.1, was enriched for day 7 colony-forming cells by a factor of 25 with respect to the starting bone marrow. In fact, 4.5% of cells in this fraction gave rise to colonies on day 7 compared with 0.17% in the starting material. By contrast, cells giving rise to nonerythroid clusters on day 2 were enriched by just a factor of 6.6. This implies that about 70% of the day 2 proliferating cells as well as 97% or 98% of the CFU-E were removed by the separation procedures. Thus, panning with YBM 34.3 followed by cell sorting with YBM 6.1 allowed a quantitive separation of late hemopoietic precursor cells (CFU-E and some day 2 proliferating cells) from the more primitive ones (day 7 GM CFC, BFU-E, and BFU-M/E). It should be noted that CFU-E and the day 2 clonogenic cells can be recovered from the panning plates by gentle pipetting.

DISCUSSION

The cell separation technique described in this article, based on differential binding with two monoclonal antibodies YBM 34.3 and YBM 6.1, allowed a high enrichment in colony-forming cells as well as a segregation of early and late progenitors. The use of YBM 34.3 in a first stage as an indirect solid phase immunoabsorbent greatly diminished sorting time.

As noted previously, the overall yield of the panning technique was 34% in cell number and 40%–60% in colony-forming cells, but since most of these losses occur during the washing and labeling procedures, the figures for the overall yield of cell sorting will be similar. The main advantage of panning is that it allows a large number of cells to be handled as compared to cell sorting. This is a worthwhile advantage, especially when attempting to enrich for a rare cell type.

It has been documented that erythroid progenitor cells at different stages of differentiation differ in their responsiveness to erythropoietin in vitro and in vivo.[26 29] While CFU-E are highly responsive, the more primitive cells (day 7 BFU-E) are much less so and require burst-promoting activity (BPA) as an essential stimulatory signal. This difference in humoral requirements in vitro may reflect a difference in cell surface receptors. Apart from the differential sensitivity to humoral factors, there has been little documentation on difference in cell surface determinants between such stages. Evidence is provided by the present work that separation of cells on the basis of their binding to YBM 34.3 resulted in a segregation between the different stages of erythroid cell differentiation. The results indicated that expression of the antigen detected by YBM 34.3 increases with differentiation along the erythroid pathway. FACS II sorting or panning of bone marrow cells with YBM 34.3 also indicated that erythroid colonies scored on day 5 represent a heterogeneous population. Some of the colonies containing only one cell type probably represent the end-stages of cell development from late BFU-E, as they expressed the antigen detected by YBM 34.3 and were therefore removed by panning. The remainder were probably derived from more primitive precursors expressing the antigen at a level below the detectable threshold of the procedure. It is likely that these would probably give rise to macroscopic erythroid colonies scored on day 7. On the other hand, all the mixed erythroid colonies scored on day 5 would represent transitional stages of development from more primitive precursors (BFU-M/E). They would give rise to colonies scored as multipotential on day 7.

The final cell suspension obtained after the two-step separation technique described here contained 58% immature cells (blasts, promyelocytes, and myelocytes) and 35% lymphocytes. The latter lacked surface IgM and Thy-1 and could represent early cells of the lymphocyte lineage. The culture methods used showed that 9.5% of the cells were able to proliferate in vitro by day 2. Therefore, only one cell in six of those classified morphologically as immature was able to proliferate in vitro. There are several possible explanations for this discrepancy, one of which might be that the in vitro culture technique does not detect all the different types of precursors (e.g., lymphocyte precursors). It is also possible that most of the day 2 clonogenic cells do not have the typical blastic appearance, as generally assumed, but may be of the granular type.[13]

In summary, the separation of primitive from late erythroid progenitors was achieved by differential binding capacity to YBM 34.3. The panning technique provides a large number of cells from adult hemopoietic tissue with a high enrichment for primitive precursors. Such a population can be used for a further purification of stem cells in combination with other surface markers.

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