Purified Primitive Human Hematopoietic Progenitor Cells with Long-Term In Vitro Repopulating Capacity Adhere Selectively to Irradiated Bone Marrow Stroma

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Summary

We enriched bone marrow cells from 10 normal individuals for primitive hematopoietic progenitors using a two-step technique, and examined resultant primitive progenitors for their in vitro long-term repopulating capacity and their ability to adhere to irradiated stroma. Immunomagnetic depletion of mature myeloid and lymphoid progenitors resulted in a lineage-negative (Lin^-) cell population. Subsequent dual-color fluorescence activated sorting of cells with low forward and vertical light scatter properties, expressing CD34 antigen (34^+) and either bearing (DR^+) or lacking (DR^-) the HLA-DR antigen, resulted in the selection of $Lin^-34^+DR^+$ and $Lin^-34^+DR^-$ cell populations. When the $Lin^-34^+DR^+$ cell fraction was cultured in a short-term methylcellulose assay, we demonstrated a 61-fold enrichment for colony forming cells (CFC) compared with undepleted bone marrow mononuclear cells. In contrast to the $Lin^-34^+DR^+$ cells, direct culture of $Lin^-34^+DR^-$ cells in short-term methylcellulose generated significantly less CFC ($p \leq 0.001$). We then compared the capacity of $Lin^-34^+DR^+$ and $Lin^-34^+DR^-$ cells to generate sustained hematopoiesis when plated in long-term bone marrow culture (LTBMC). When LTBMC were initiated with plated $Lin^-34^+DR^+$ cells, we recovered high numbers of CFC during the first week, but observed a rapid decline in the number of harvested CFC over the following weeks. No CFC could be recovered after week 7. In contrast, LTBMC initiated with plated $Lin^-34^+DR^-$ cells yielded significantly greater numbers of CFC than LTBMC initiated with plated $Lin^-34^+DR^+$ cells ($p \leq 0.001$), and this was sustained for at least 12 wk of culture. The $Lin^-34^+DR^+$ population was only 6.6-fold enriched for primitive progenitors capable of initiating and sustaining hematopoiesis in LTBMC when compared with undepleted bone marrow mononuclear cells, while the $Lin^-34^+DR^-$ population was 424-fold enriched for such primitive progenitors ($p \leq 0.001$). Finally, we examined the capacity of both $Lin^-34^+DR^+$ and $Lin^-34^+DR^-$ populations to adhere to irradiated allogeneic stroma. We used a previously described "panning method" in which cells are plated onto stroma for 2 h, the nonadherent cells removed by extensive washing, and the adherent fraction maintained under conditions favoring LTBMC growth. When stroma was panned with $Lin^-34^+DR^+$ cells, 79 ± 10% of the cells were recovered in the panning effluent. In contrast, when stroma was panned with $Lin^-34^+DR^-$ cells, significantly fewer (37 ± 7%) ($p \leq 0.001$) cells were recovered in the panning effluent. Unlike LTBMC initiated with plated $Lin^-34^+DR^+$ cells, virtually no CFC were recovered from LTBMC initiated with panned $Lin^-34^+DR^+$ cells. In contrast, LTBMC initiated with either plated or panned $Lin^-34^+DR^-$ cells generated high numbers of CFC for a minimum of 12 wk. These studies present the first evidence that further purification of $34^+/DR^-$ cells using an additional immunomagnetic depletion of committed myeloid and lymphoid progenitors results in a $Lin^-34^+DR^-$ population that is significantly enriched (424-fold) for primitive progenitors capable of initiating and sustaining growth of committed myeloid progenitors in LTBMC for at least 12 wk. These studies also provide the first evidence that primitive progenitors capable of adhering avidly to irradiated bone marrow–derived stroma when panned for 2 h are present exclusively in the $Lin^-34^+DR^-$ population. In contrast, $Lin^-34^+DR^+$ cells, which are committed clonogenic precursors, do not exhibit the ability to adhere to irradiated stroma. Further study of these cell populations will allow detailed analysis of interactions between primitive hematopoietic stem cells and the bone marrow microenvironment.

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The characteristics of the most primitive human hematopoietic progenitor cells required to initiate and sustain hematopoiesis in vivo are not well understood. Standard culture systems using semisolid media detect clonogenic cells but fail to support the growth of more immature progenitor cells (1–3). The long-term bone marrow culture (LTBMC)[1] system described by Dexter et al. (4) is an in vitro model that closely mimics the in vivo hematopoietic environment and supports proliferation of more primitive progenitor cells. It is believed that immature progenitor cells lodge and proliferate in the adherent stromal layer and are released into the overlying supernatant as differentiation proceeds (5).

Cells capable of complete hematopoietic reconstitution are found in the CD34 antigen–positive subfraction of bone marrow (BM) (6, 7). Several recent studies demonstrate that clonogenic cells may be distinguished from their more primitive progenitors by their chemosensitivity and expression of cell surface antigens. Clonogenic cells are sensitive to treatment with several S-phase inhibitors, such as 5-fluorouracil (8–10), hydroxyurea (9, 10), and 4-hydroperoxycyclophosphamide (11), implying that these progenitors are actively cycling. Clonogenic cells express CD34 antigens (9, 11–13) on the cell membrane in association with either CD33 (12) and/or HLA-DR (9, 13–15) antigens. The more primitive progenitor cells that are capable of generating CFU-Blast in semisolid culture systems (9, 16, 17), which produce BL-CFC on stromal feeder layers (10, 11, 18) and which have the capacity of initiating long-term cultures (12, 13), are quiescent, chemoinsensitive cells. These small blast-like cells express CD34 (9, 12, 13, 16–19) antigens on the cell membrane but lack HLA-DR (9, 15, 16) and/or CD33 antigen (12) expression.

We describe a two-step technique that enriches primitive human hematopoietic BM progenitor cell populations. The enrichment procedure uses a negative immunomagnetic depletion that removes cells of committed myeloid and lymphoid lineage (Lin^-). Subsequently, a positive fluorescence-activated cell selection is performed that enriches for cells bearing the CD34 antigen (34^+) and further selects for cells either bearing the HLA-DR antigen (DR^+) or lacking this antigen (DR^-). Our study confirms the observation that $34^+/DR^-$ cells, but not the $34^+/DR^+$ population, initiate committed myeloid progenitor growth in LTBMC. We demonstrate that additional purification of the $34^+/DR^-$ population by depletion of committed progenitors results in a population that is highly enriched for LTBMC-initiating cells and sustains growth of committed progenitors in LTBMC for at least 3 mo. This study also demonstrates that primitive LTBMC-initiating cells exclusively present in the $Lin^-34^+DR^-$ population are capable of adhering to irradiated bone marrow–derived stroma, while committed myeloid progenitors in the $Lin^-34^+DR^+$ population do not exhibit the ability to adhere to stroma.

[1] *Abbreviations used in this paper:* BM, bone marrow; BMMNC, bone marrow mononuclear cells; CFC, colony-forming cells; E, erythroid; GM, granulocyte/macrophage; LTBMC, long-term bone marrow cultures.

Materials and Methods

Marrow Samples

BM samples were obtained from 19 healthy individuals by aspiration of small volumes from the posterior iliac crest. BM cells were collected in preservative-free heparin (Solopak Laboratories, Franklin Park, IL). BM cells were depleted of RBC and granulocytes by density gradient centrifugation over Histopaque-1077 (Sigma Chemical Co., St. Louis, MO) at 400 *g* for 30 min at room temperature. Informed consent was obtained using guidelines approved by the Committee on the Use of Human Subjects at the University of Minnesota.

Purification of BM Subpopulations

To obtain purified progenitor cell populations from BMMNC, we used a two-step purification protocol. After an initial negative immunomagnetic selection into a Lineage negative (Lin^-) population (step 1), cells were selected positively for their small hypogranular morphology and expression of CD34 and HLA-DR antigens using four-parameter FACS (step 2) in $Lin^-34^+DR^+$ and $Lin^-34^+DR^-$ cell fractions.

Immunomagnetic Depletion. BMMNC were depleted of committed myeloid cells, monocytes, and lymphoid progenitors by immunomagnetic separation. All preparations were done on ice using cold media to minimize nonspecific binding. BMMNC were incubated for 30 min at 4°C, with a mixture of mAbs with overlapping specificity for T and B lymphocytes (5 $\mu g/10^6$ cells), NK, monocyte, and myeloid lineage, including anti-CD2 (Leu-5), anti-CD3 (Leu-4), anti-CD16 (Leu-11), anti-CD11b (Leu-15), anti-CD15 (My-18), anti-CD19 (Leu-12), anti-CD56 (Leu-19), and anti-CD71 (transferrin receptor) mAbs (Becton Dickinson & Co., Mountain View, CA). Cells were washed twice and were then incubated with immunomagnetic beads coated with goat anti–mouse IgG and IgM (Advanced Magnetics Inc., Cambridge, MA) for 30 min at 4°C with periodic agitation (4×10^7 beads were used per 10^6 cells). Rosettes and free particles were then removed using a BioMag Separator (Advanced Magnetics Inc.). The rosette-free fraction was inspected for the presence of rosettes and, if necessary, the above procedure was repeated once or twice. The rosette-positive fraction was washed twice and nonrosetted cells were recovered. The rosette-free suspension was called "lineage-negative" BMMNC (Lin^-).

In selected cases, BMMNC were depleted only of cells expressing CD2, CD11b, CD15, and CD19. Procedures used for this less extensive depletion were similar to the above described methods. The rosette-free fraction obtained after this alternative depletion will be called "partial Lineage negative" (part Lin^-).

To assess the effectiveness of the immunomagnetic depletion, BMMNC and Lin^- cells were analyzed for the presence of cells bearing the CD2, CD3, CD11b, CD15, CD16, CD19, CD56, and CD71 antigens. Lin^- cells obtained after immunomagnetic separation were first incubated with 100 μg goat $F(ab)'_2$ anti–mouse IgG + IgM (Tago Inc., Burlingame, CA) to block any mouse IgG mAb still present after immunomagnetic depletion. The cells were next incubated with mouse serum (Sigma Chemical Co.) to block any unbound active site on the goat $F(ab)'_2$ anti–mouse IgG + IgM. BMMNC and Lin^- cells were then incubated with saturating amounts of anti-CD2, anti-CD11b, anti-CD15, anti-CD71 antibodies for 30 min on ice. Cells were washed and incubated with saturating amounts of PE-conjugated goat $F(ab)'_2$ anti–mouse IgG + IgM (Tago Inc.) for 30 min and washed twice. Control stains were performed by labeling cells with isotype-matched mouse IgG or IgM followed by PE-conjugated goat $F(ab)'_2$ anti–mouse IgG

+ IgM in each experiment. Alternatively, BMMNC or Lin^- cells were incubated with saturating amounts of anti-CD3 FITC, anti-CD16 PE, anti-CD19 PE, or anti-CD56 PE for 30 min on ice. Cells were then washed twice. Control stains with isotype-matched PE- or FITC-coupled mouse IgG or IgM were included in each experiment.

FACS. For cell labeling, mouse anti-CD34 (HPCA-1) and mouse anti-HLA-DR mAbs were used (Becton Dickinson & Co.). Lin^- cells obtained after immunomagnetic separation were first incubated with 100 μg goat $F(ab)'_2$ anti-mouse IgG (Tago Inc.) to block any mouse IgG mAb still present after immunomagnetic depletion. The cells were next incubated with 500 μg mouse IgG (Sigma Chemical Co.) to block any unbound active site on the goat $F(ab)'_2$ anti-mouse IgG. Cells were incubated with 25 μg of anti-CD34 antibody/10^6 cells for 30 min at 4°C and washed twice. We then treated the cells with 25 μg/10^6 cells FITC-conjugated goat $F(ab)'_2$ anti-mouse IgG (Tago Inc.) for 30 min at 4°C and washed twice. To block any unbound active site on the goat $F(ab)'_2$ anti-mouse IgG, we then incubated the cells with 500 μg mouse IgG. The cells were then stained with 25 μg anti-HLA-DR antibody/10^6 cells for 30 min and washed twice. We finally incubated the cells with PE-conjugated goat $F(ab)'_2$ anti-mouse IgG (25 μg/10^6 cells) (Tago Inc.) for 30 min and washed the cells twice. Negative control stains for CD34/FITC and DR/PE using isotype-matched mouse IgG followed by PE- and FITC-conjugated goat $F(ab)'_2$ anti-mouse IgG were included in each experiment.

Cells were sorted on a FACS-Star laser flow cytometry system (Becton Dickinson & Co.) equipped with a cosort 40 computer. Sorting windows were established for four separate parameters: forward and vertical light scatter, FITC, and PE fluorescence. A first selection consisted of gating in for cells with low vertical and very low/low horizontal light scatter properties. The sorting gates were then set to isolate cells expressing high-density CD34-FITC antigen and either the presence ($Lin^-34^+DR^+$ subpopulation) or absence ($Lin^-34^+DR^-$ subpopulation) of HLA-DR-PE. The $Lin^-34^+DR^+$ fraction contained >90% $CD34^+$ and >90% $HLA\text{-}DR^+$ cells, while the $Lin^-34^+DR^-$ fraction contained >90% $CD34^+$ cells and <5% $HLA\text{-}DR^+$ cells.

Short-term Methylcellulose Assay.

Bone marrow mononuclear cells (BMMNC), Lin^-, $Lin^-34^+DR^+$, and $Lin^-34^+DR^-$ cells were plated in methylcellulose (final concentration, 1.12%) (Fisher Scientific Co., Pittsburgh, PA) with IMDM (Gibco Laboratories, Grand Island, NY) supplemented with 30% FCS (HyClone Laboratories, Logan, UT), antibiotics (penicillin [1,000 U/ml] and streptomycin [100 U/ml]; Gibco Laboratories), 5×10^{-5} M 2-ME, 3 IU recombinant erythropoietin (Epoetin) (Amgen, Thousand Oaks, CA), and 10% conditioned media from the bladder carcinoma cell line 5637. Cultures were incubated in a humidified atmosphere at 37°C and 5% CO_2 for 14–21 d. The cultures were then assessed for the presence of CFU-MIX, granulocyte/macrophage CFU(CFU-GM), and erythroid BFU(BFU-E) as previously described (20). Progenitor enrichment was calculated as the number of colonies present in cultures with subpopulations of BMMNC/10^6 plated cells, divided by the number of colonies in cultures with undepleted BMMNC/10^6 plated cells. The percent recovery of clonogenic cells was then calculated as the percentage of all cells recovered in the fraction multiplied by the calculated enrichment factor for the subpopulation. Likewise, cells harvested weekly from the adherent and nonadherent layers of LTBMC were plated in short-term methylcellulose culture, and CFU-MIX, CFU-GM, and BFU-E were enumerated at day 14–16 of cultures, as described above, in order to determine the capability of plated and panned cells to initiate and sustain hematopoiesis.

Long-term Marrow Cultures

Plating. BMMNC, Lin^-, $Lin^-34^+DR^+$, and $Lin^-34^+DR^-$ cell populations were cultured in a long-term culture system as follows. Normal allogeneic stromal layers were cultured in T25 flasks (Corning Glass Works, Corning, NY) for 14–28 d, as previously described (4), and irradiated at 400 cGy/min with a MARK 1 Cesium irradiator (Shepard and Associates, Glendale, CA) to provide a dose of 1,000 cGy 5–7 d after irradiation. Stromal cells were recovered by treatment of the adherent layers with 0.1% collagenase (Worthington Biochemical Corp., Freehold, NJ) for 2–3 h. The stromal cells were then subcultured at 0.6×10^6/ml in 24-well plates (Costar, Cambridge, MA). BMMNC (10^6/well), Lin^- (10^5/well), $Lin^-34^+DR^+$ (10^4/well), and $Lin^-34^+DR^-$ (10^4/well) cells were then plated on allogeneic irradiated stroma in 1 ml of LTBMC media (IMDM with 12.5% FCS, 12.5% horse serum [HyClone Laboratories, Logan, UT], 2 mM L-glutamine, 1,000 U/m penicillin, and 100 U/ml streptomycin [Gibco Laboratories]) and 10^{-6} M hydrocortisone [A-Hydrocort; Abbott Laboratories, North Chicago, IL).

Panning. Alternatively, $Lin^-34^+DR^+$ and $Lin^-34^+DR^-$ cell populations were panned on allogeneic irradiated stroma in LTBMC media for 2 h, the stroma was washed extensively with warm IMDM, and the nonadherent cells were recovered in the panning effluent (10, 18). The cells in the panning effluent were enumerated by either counting in a hemocytometer, or by reanalysis of the cell population by flow cytometry for the presence of $Lin^-34^+DR^+$ or $Lin^-34^+DR^-$ cells.

Maintenance of Cultures. All LTBMC cultures were maintained in a humidified atmosphere at 37°C with 5% CO_2. At weekly intervals, the cultures were fed by removing half of the supernatant and replacing it with fresh media. Nonadherent cells recovered in the supernatant, as well as adherent cells recovered from selected stromal layers after treatment with 0.1% collagenase, were assayed weekly in a short-term methylcellulose assay for the presence of colony-forming cells (CFC).

Statistical Analysis

Results of experimental points obtained from multiple experiments are reported as the mean ± 1 SEM.

Results

Enrichment by Immunomagnetic Depletion for Lineage$^-$ (Lin^-) BM Cells. To obtain marrow cells enriched for primitive progenitors, we first performed a negative immunomagnetic bead selection of BMMNC with a cocktail of mAbs against T (CD2, CD3) and B lymphocytes (CD19), NK cells (CD2, CD16, CD56), monocytes, and more mature myeloid marrow elements (CD11b, CD15), as well as erythroid progenitors and some CFU-GM (CD71). This Lin^- subpopulation contained 7.9 ± 0.58% of the initial BMMNC number (n = 12). This initial depletion resulted in a >95% removal of cells expressing CD2, CD3, CD11b, CD15, CD16, CD19, CD56, or CD71 antigens. Assessment of the Lin^- subpopulation for the presence of CFC by direct culture in a short-term methylcellulose assay yielded 1,010 ± 39 CFC/10^5 plated

Table 1. *Enrichment and Recovery of Clonogenic Progenitors*

BM populations	*n*	Percent cells	CFC/10^5 cells	Enrichment	Percent recovery
BMMNC	12	-	100 ± 25	-	-
Lin^-	12	7.9 ± 0.58	1,010 ± 31	6.3 ± 1.4	58.6 ± 6.7
$Lin^-34^+DR^+$	12	0.65 ± 0.06	7,860 ± 860	61.3 ± 9.5	28.9 ± 4.7
$Lin^-34^+DR^-$	12	0.24 ± 0.02*	2,130 ± 250*	14.9 ± 3.2*	6.3 ± 1.1*

* Comparison between $Lin^-34^+DR^+$ and $Lin^-34^+DR^-$ cells; $p \leq 0.001$.

Lin^- cells (n = 12), or a 6.3 ± 1.4-fold enrichment over undepleted BMMNC (Table 1). When the Lin^- cells were plated in LTBMC and adherent and nonadherent layers harvested weekly thereafter, we observed sustained generation of CFC in both adherent and nonadherent layers of LTBMC initiated with plated Lin^- cells for at least 9 wk.

Enrichment by FACS of Primitive Progenitors. Further purification of progenitors was achieved by sorting the Lin^- population into $Lin^-34^+DR^+$ and $Lin^-34^+DR^-$ subpopulations according to their light scatter properties and their expression of the CD34 and HLA-DR antigens on the cell membrane. Cells with low vertical and low/very low horizontal light scatter properties present in the "blast window" were selected (window A) (Fig. 1). The blast window contained 34.9 ± 2.1% of all Lin^- cells (n = 12). 79 ± 1.3% of cells present in window A expressed the CD34 antigen on the cell membrane (n = 12). Sorting gates were then set to purify further cells from window A for cells expressing high density of CD34-FITC antigen and either absence (window B, $Lin^-34^+DR^-$ fraction) or presence (window C, $Lin^-34^+DR^+$ fraction) of HLA-DR PE antigen. The $Lin^-34^+DR^+$ fraction contained 49 ± 2.4% of the cells present in window A (n = 12), or 0.65 ± 0.06% of cells present in the initial BMMNC, and consisted mainly of cells with the appearance of small undifferentiated blasts. The $Lin^-34^+DR^-$ fraction comprised only 23.4 ± 0.9% of cells present in window A (n = 12; $p \leq 0.001$), or 0.24 ± 0.02% of the original undepleted low density marrow cells ($p \leq 0.001$), and were morphologically smaller than cells in the $Lin^-34^+DR^+$ fraction.

Growth of Enriched Primitive Progenitor Cells in Short-term Methylcellulose Assay. To assess the presence of committed hematopoietic progenitor cells in the sorted subpopulations, sorted cells (10^3/ml) were plated in short-term methylcellulose assay. Culture in short-term methylcellulose assay of the sorted $Lin^-34^+DR^+$ cells yielded 7,860 ± 860 CFC/10^5 $Lin^-34^+DR^+$ plated cells (n = 12) (Table 1). This represents an enrichment of 61.3 ± 9.5-fold compared with undepleted BMMNC (Table 2). The total recovery of CFC in this Lin^-34^+ DR^+ fraction was, however, only 28.9 ± 4.7% of the number of CFC present in undepleted BMMNC (Table 1).

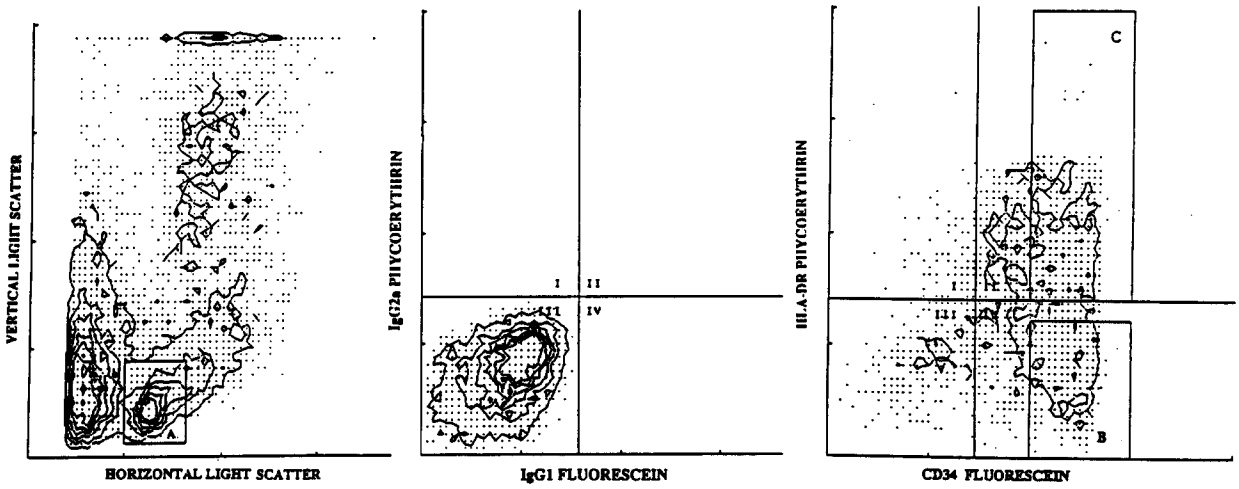

Figure 1. Representative analysis of light scatter properties (*left*) and distribution of HLA-DR and CD34 antigen (*right*) on lineage-negative BMMNC analyzed by two-color immunofluorescence. Cells with low/very low horizontal and low vertical light scatter properties (window *A*) represent 21.5% of the total Lin^- BMMNC population (*left*). Cells present in the sorting window based on vertical and horizontal light scatter properties were analyzed for their reactivity with the mAbs HLA-DR-PE and CD34-FITC (*right*). Quadrant III (*middle*) represents double-negative cells based on control stains with isotype-matched mouse IgG for both HLA-DR-PE and CD34-FITC. $Lin^-34^+DR^-$ cells present in window *B* (HLA-DR antigen negative and high expression of CD34 antigen) represent 5.1% of the Lin^- population. $Lin^-34^+DR^+$ cells present in window *C* (HLA-DR antigen positive and high expression of CD34 antigen) represent 8.9% of the initial Lin^- population.

In contrast to the $Lin^-34^+DR^+$ fraction, only 2,130 ± 250 CFC/10^5 plated cells (n = 12; $p \leq 0.001$) were recovered in short-term methylcellulose assays of $Lin^-34^+DR^-$ cells. The calculated enrichment over undepleted BMMNC of 14.9 ± 3.2 (n = 12; $p \leq 0.001$)-fold, and the total recovery of CFC in the $Lin^-34^+DR^-$ fraction of 6.3 ± 1.1% (n = 12; $p \leq 0.001$) of the CFC present in the initial BMMNC, were also significantly lower then those of the $Lin^-34^+DR^+$ population (Table 1). These data indicate that the $Lin^-34^+DR^+$ cells contain significantly higher numbers of committed hematopoietic progenitors than the $Lin^-34^+DR^-$ subpopulation.

We compared the presence of CFC in the $Lin^-34^+DR^+$ and $Lin^-34^+DR^-$ populations with control progenitor cell populations obtained after less extensive immunomagnetic depletion (using anti-CD2, anti-CD11b, anti-CD15, and anti-CD19, but not anti-CD16, anti-CD56, and anti-CD71 mAbs) followed by positive FACS selection for CD34 and HLA-DR antigens, referred to as part-$Lin^-34^+DR^+$ and part-$Lin^-34^+DR^-$ cells. Less extensive depletion of myeloid and lymphoid precursors in the immunomagnetic depletion step resulted in a significantly greater number of CFC present in both the part-$Lin^-34^+DR^+$ population (17,940 ± 2,530 CFC/10^5 plated part-$Lin^-34^+DR^+$ cells; n = 7; $p \leq 0.001$) and part-$Lin^-34^+DR^-$ population (3,990 ± 950 CFC/10^5 plated part-$Lin^-34^+DR^-$ cells; n = 7; $p \leq 0.01$) compared with the above described $Lin^-34^+DR^+$ and $Lin^-34^+DR^-$ cell fractions (Table 2). Comparison of either $Lin^-34^+DR^+$ with part-$Lin^-34^+DR^+$ or $Lin^-34^+DR^-$ with part-$Lin^-34^+DR^-$ populations demonstrated that both single-lineage and multi-lineage colonies are depleted more extensively when a broader panel of antibodies is used for the initial immunomagnetic depletion step (Table 2).

Growth of Plated Primitive Progenitor Cells in LTBMC. We then compared the capacity of $Lin^-34^+DR^+$ and $Lin^-34^+DR^-$ populations to initiate and sustain hematopoiesis in LTBMC by plating and culturing both cell fractions on preestablished irradiated stroma. Nonadherent and adherent cells were harvested weekly and replated in short-term methylcellulose assay. At day 14–16 of cultures, methylcellulose cultures were assessed for the presence of single and multilineage CFC.

$Lin^-34^+DR^+$ cells plated in LTBMC generated high numbers of CFC in the nonadherent fraction at week 1 of culture (5,142 ± 1,003 CFC/10^6 plated $Lin^-34^+DR^+$ cells; n = 10) (Fig. 2 *A*). However, there was a progressive decline in CFC harvested from the nonadherent cell fraction from week 1 through week 7. No CFC were recovered from the nonadherent fraction of LTBMC initiated with plated $Lin^-34^+DR^+$ cells after week 7 (n = 6) (Fig. 2 *A*), resulting in a cumulative recovery of 10,079 ± 2,012 CFC/10^6 plated $Lin^-34^+DR^+$ cells over the entire 12-wk period (n = 3) (Fig. 3 *A*).

Adherent layers from LTBMC initiated with $Lin^-34^+DR^+$ cells were harvested weekly, and the harvested cell population was plated in short-term methylcellulose assay. Sequential analysis of adherent layers from LTBMC initiated with $Lin^-34^+DR^+$ cells for the presence of CFC demonstrated a similar pattern of recovery of CFC as was demonstrated for nonadherent layers of the corresponding cultures (Fig. 2 *B*). At 1 wk, 11,861 ± 2,479 CFC/10^6 plated $Lin^-34^+DR^+$ cells were recovered from harvested adherent layers of LTBMC initiated with $Lin^-34^+DR^+$ cells (n = 7). There was, however, a very steep drop-off in CFC recovered from these adherent layers over the next 3 wk, and CFC could not be found after week 5 (n = 6). More detailed analysis of the type of clonogenic cells present in both adherent and nonadherent layers of LTBMC initiated with plated $Lin^-34^+DR^+$ cells revealed that BFU-E and CFU-GM were present for 5 wk (adherent) (n = 10) and 6 wk (nonadherent) (n = 6), while multi-lineage CFU-MIX were recovered for only 3 wk (adherent and nonadherent) (n = 10),

Table 2. *Recovery of Clonogenic Progenitors after Partial and Extensive Immunomagnetic Depletion of Committed Myeloid and Lymphoid Progenitor Cells*

BM population	n	Percent cells	Colonies/10^5 cells			
			All colonies	BFU-E	CFU-MIX	CFU-GM
$Lin^-34^+DR^+$*	12	0.65 ± 0.06	7,860 ± 860	2,270 ± 520	370 ± 60	5,910 ± 610
part-$Lin^-34^+DR^+$‡	7	2.1 ± 0.39§	17,940 ± 2,530§	5,300 ± 1,250‖	1,380 ± 190§	11,040 ± 1,390§
$Lin^-34^+DR^-$*	12	0.24 ± 0.02	2,130 ± 250	870 ± 11	127 ± 29	1,170 ± 180
part-$Lin^-34^+DR^-$‡	7	0.68 ± 0.17§	3,990 ± 950‖	1,520 ± 350¶	167 ± 59**	2,640 ± 570§

* $Lin^-34^+DR^+$ or $Lin^-34^+DR^-$ are obtained after depletion of CD2, CD3, CD11b, CD15, CD16, CD19, CD56, CD71 positive cells followed by four-parameter FACS sorting, as described in Materials and Methods.
‡ Part-$Lin^-34^+DR^+$ and part-$Lin^-34^+DR^-$ cells are obtained after depletion of CD2, CD11b, CD15, CD16, CD19 positive cells followed by four-parameter FACS sorting, as described in Materials and Methods.
§ $p \leq 0.001$.
‖ $p \leq 0.01$.
¶ $p \leq 0.05$.
** p = NS.

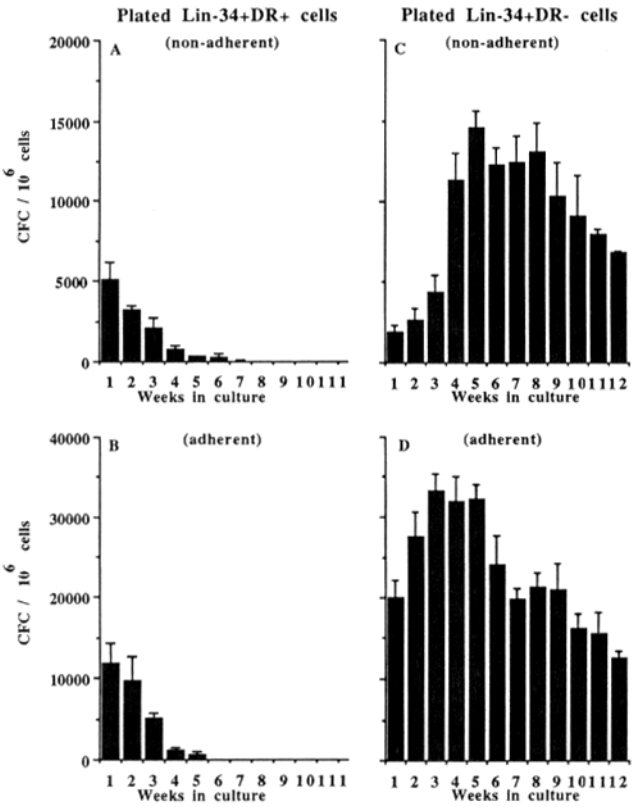

Figure 2. Lineage-negative BMMNC were separated by FACS according to low/very low horizontal and low vertical light scatter properties into Lin$^-$34$^+$DR$^+$ cells (*A* and *B*) and Lin$^-$34$^+$DR$^-$ cells (*C* and *D*). (*Top*) CFC recovered from nonadherent layers of LTBMC initiated with plated Lin$^-$34$^+$DR$^+$ cells (*A*) and plated Lin$^-$34$^+$DR$^-$ cells (*C*). (*Bottom*) CFC recovered from adherent layers of LTBMC initiated with plated Lin$^-$34$^+$DR$^+$ cells (*B*) and plated Lin$^-$34$^+$DR$^-$ cells (*D*). Data points are CFC recovered from weekly harvested nonadherent and adherent layers of LTBMC per 10^6 plated cells used to initiate the LTBMC. For nonadherent layers, data points between weeks 1 and 5 represent mean ± SEM of 10 experiments; data points between weeks 6 and 8 represent mean ± SEM of six experiments; data points between weeks 9 and 12 represent mean ± SEM of three experiments. For adherent layers, data points between weeks 1 and 3 represent mean ± SEM of seven experiments; data points at week 4–5 represent mean ± SEM of 10 experiments, data points between weeks 6 and 8 represent mean ± SEM of six experiments; data points between weeks 9 and 12 represent mean ± SEM of three experiments.

suggesting that the Lin$^-$34$^+$DR$^+$ population contains only relative mature hematopoietic progenitor cells (Fig. 4, *A–C*).

It has been demonstrated that committed clonogenic cells have disappeared from LTBMC cultures by week 4, and that the number of CFC recovered from LTBMC after 4 wk of culture represent the number of more immature progenitors present in the initiating cell population (13, 20, 21, 22). We calculated the enrichment and recovery of cells capable of initiating and sustaining hematopoiesis in LTBMC as the total number of CFC recovered from nonadherent and adherent layers 5 wk after initiation of the LTBMC with enriched BM cell populations divided by the total number of CFC recovered from nonadherent and adherent layers of LTBMC initiated with undepleted BMMNC. The enrichment for cells capable of initiating LTBMC in the Lin$^-$34$^+$DR$^+$ cell population is 6.6 ± 2.4-fold (n = 10) (Table 3).

Similar experiments were performed to examine the capacity of the Lin$^-$34$^+$DR$^-$ subpopulation to initiate and sustain hematopoiesis when plated on irradiated stroma. Cells from nonadherent and adherent layers of LTBMC initiated with Lin$^-$34$^+$DR$^-$ cells were analyzed weekly for the presence of CFC (Fig. 2).

Plating of Lin$^-$34$^+$DR$^-$ cells in LTBMC (Fig. 2 *C*) resulted in a significantly lower yield of CFC in the nonadherent fraction for the first week of culture compared with LTBMC initiated with plated Lin$^-$34$^+$DR$^+$ cells (Fig. 2 *A*) (5,142 ± 1,003 CFC/10^6 plated Lin$^-$34$^+$DR$^+$ cells [n = 10] vs. 1,918 ± 419 CFC/10^6 plated Lin$^-$34$^+$DR$^-$ cells [n = 10]; p = 0.009). In contrast to LTBMC initiated with Lin$^-$34$^+$DR$^+$ cells, the number of CFC harvested from nonadherent layers of LTBMC initiated with plated Lin$^-$34$^+$DR$^-$ cells increased progressively after week 3 of culture, and a maximal recovery of 14,612 ± 1,008 CFC/10^6 plated Lin$^-$34$^+$DR$^-$ cells was achieved during week 5 (n = 10) ($p \leq 0.001$). At week 12 of culture, 6,800 ± 100 CFC/10^6 plated Lin$^-$34$^+$DR$^-$ cells could still be found in the nonadherent fraction of LTBMC initiated with plated Lin$^-$34$^+$DR$^-$ cells (n = 3) (Fig. 2 *C*), resulting in a cumulative recovery of 103,474 ± 4,135 CFC/10^6 plated Lin$^-$ 34$^+$ DR$^-$ cells over the entire 12-wk period (n = 3) (Fig. 3 *B*).

A significantly greater number of CFC was recovered from adherent layers of LTBMC initiated with plated Lin$^-$34$^+$ DR$^-$ cells (Fig. 2 *D*) compared with LTBMC initiated with Lin$^-$34$^+$DR$^+$ cells (Fig. 2 *B*) at any time point (week 1, p = 0.026, n = 7; week 2, p = 0.002, n = 4; week 3, $p \leq 0.001$, n = 10). After an initial increase in CFC recovered from adherent layers of LTBMC initiated with plated Lin$^-$34$^+$DR$^-$ cells, a maximal recovery of 33,876 ± 1,778 CFC/10^6 plated Lin$^-$34$^+$DR$^-$ cells at 5 wk of culture was observed (n = 10) ($p \leq 0.001$). After week 5 of culture, a progressive decrease in CFC recovered from the adherent fraction of LTBMC initiated with plated Lin$^-$34$^+$DR$^-$ cells occurred. At week 12 of culture, 12,650 ± 425 CFC/10^6 plated Lin$^-$34$^+$DR$^-$ cells could still be recovered from adherent layers of LTBMC initiated with plated Lin$^-$34$^-$DR$^-$ cells (n = 3) (Fig. 2 *D*). Unlike LTBMC initiated with plated Lin$^-$34$^+$DR$^+$ cells, LTBMC initiated with plated Lin$^-$34$^+$ DR$^-$ cells gave rise to a sustained growth of both multilineage clonogenic progenitors (MIX-CFU) and single-lineage colonies (GM-CFU and E-BFU) (Fig. 4, *D–F*).

The enrichment over undepleted BMMNC for primitive progenitors capable of initiating and maintaining hematopoiesis in vitro in this Lin$^-$34$^+$DR$^-$ fraction (424 ± 37-fold; n = 10; Table 3) was significantly greater than that for the Lin$^-$34$^+$DR$^+$ fraction ($p \leq 0.001$). The total recovery of such cells in the Lin$^-$34$^+$DR$^-$ fraction was 68 ± 9.5% ($p \leq 0.001$) of the initial BMMNC (Table 3).

These data demonstrate that the Lin$^-$34$^+$DR$^-$ subpopulation is highly enriched for immature progenitor cells capable of initiating and sustaining generation of multi- and single-lineage CFC when plated in LTBMC. In contrast, the

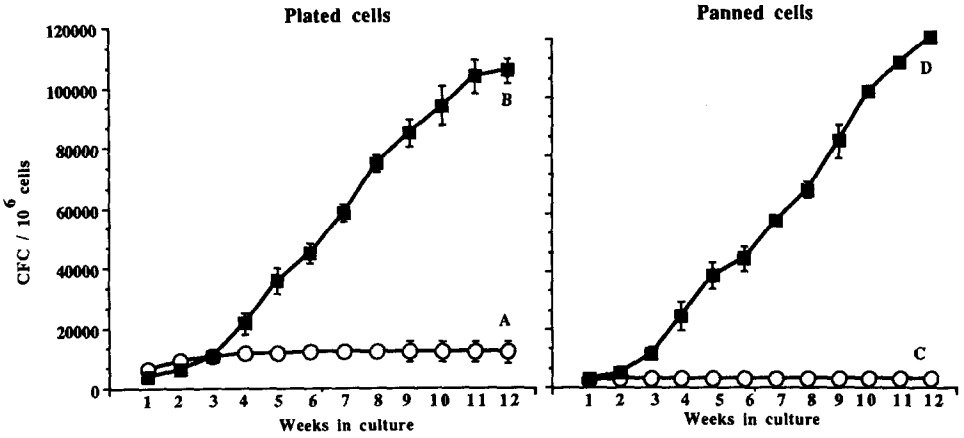

Figure 3. Lineage-negative BMMNC were separated by FACS according to low/very low horizontal and low vertical light scatter properties into Lin⁻34⁺DR⁺ cells (*A* and *C*) and Lin⁻34⁺DR⁻ cells (*B* and *D*). Data points are cumulative numbers of CFC recovered from weekly harvested nonadherent layers of LTBMC per 10^6 plated or panned cells used to initiate the LTBMC. The left panel represent cumulative numbers of CFC harvested from LTBMC initiated with plated Lin⁻34⁺DR⁺ cells (*A*) and plated Lin⁻34⁺DR⁻ cells (*B*). The right panel represents cumulative numbers of CFC harvested from LTBMC initiated with panned Lin⁻34⁺DR⁺ cells (*C*) or panned Lin⁻34⁺DR⁻ cells (*D*). Data points between weeks 1 and 5 represent mean ± SEM of 10 experiments; data points between weeks 6 and 8 represent mean ± SEM of six experiments; data points between weeks 9 and 12 represent mean ± SEM of three experiments.

Lin⁻34⁺DR⁺ fraction contains only a few cells capable of generating short-term production of predominantly single-lineage CFC when plated in LTBMC.

Growth of Panned Primitive Progenitor Cells In LTBMC. To determine the capacity of progenitor cell populations to adhere to stroma, we panned preestablished, irradiated, allogeneic stromal layers with Lin⁻34⁺DR⁺ or Lin⁻34⁺ DR⁻ cells. The cells were plated onto stroma in hydrocortisone-containing LTBMC medium and incubated at 37°C with 5% CO_2 for 2 h. The nonadherent cells were then extensively washed off with warm IMDM, and the adherent cell fraction was maintained under conditions promoting growth of LTBMC (8, 14). As in the previous experiments, we analyzed weekly the nonadherent and adherent layers for the presence of CFC.

When Lin⁻34⁺DR⁺ cells were panned on stromal layers, 79.14 ± 10.2% (n = 10) of the cells were recovered in the panning effluent, as determined either by enumerating the cells in a hemocytometer or by reanalysis by FACS of the cells present in the panning effluent (Table 4). LTBMC initiated with panned Lin⁻34⁺DR⁺ cells generated only a few CFC in the nonadherent layer for 5 wk (Fig. 5 *A*). This resulted in a significantly lower cumulative number of CFC (604 ± 391 CFC/10^6 Lin⁻34⁺DR⁺ panned cells) (n = 3) recovered from LTBMC initiated with panned Lin⁻34⁺ DR⁺ cells over a 12-wk period, compared with LTBMC initiated with plated Lin⁻34⁺DR⁺ cells (10,079 ± 2,012 CFC/10^6 Lin⁻34⁺DR⁺ cells) (n = 3) ($p \leq 0.001$) (Fig. 3 *C*). Weekly culture in short-term methylcellulose assay of cells from adherent layers of LTBMC with panned Lin⁻34⁺ DR⁺ cells revealed the presence of only a few CFC during the first 3 wk of culture, and no CFC were recovered after week 3 (n = 10) (Fig. 5 *B*).

When stroma was panned with Lin⁻34⁺DR⁻ cells, only 37 ± 2.05% (n = 10) of the cells were recovered in the panning effluent, which was significantly lower than the recovery of cells after panning stroma with Lin⁻34⁺DR⁺ cell ($p \leq 0.001$) (Table 4). Analysis of cells harvested weekly from the nonadherent fractions of LTBMC initiated with panned Lin⁻34⁺DR⁻ cells (Fig. 5 *C*) revealed a similar pattern of recovery of CFC, as observed in LTBMC initiated with plated Lin⁻34⁺DR⁻ cells. During the first week, a slightly lower number of CFC was recovered from nonadherent layers of LTBMC with panned Lin⁻34⁺DR⁻ cells (Fig. 5 *C*) than from LTBMC with plated Lin⁻34⁺DR⁻ cells (Fig. 2 *C*) (1,908 ± 419 CFC/16 plated Lin⁻34⁺DR⁻ cells [n = 10] vs. 946 ± 310 CFC/10^6 panned Lin⁻34⁺DR⁻ cells [n = 10]; p = 0.084). From week 3, there was a steep increase in number of CFC recovered from the nonadherent fraction, with a maximal recovery of 15,608 ± 1,725 CFC/10^6 panned Lin⁻34⁺DR⁻ cells 5 wk after initiation of the LTBMC (n = 10). At week 12 of culture, 8,350 ± 1,375 CFC/10^6 panned Lin⁻34⁺DR⁻ cells (n = 3) could be harvested from the nonadherent cell fraction of LTBMC initiated with panned Lin⁻34⁺DR⁻ cells (Fig. 5 *C*), resulting in a cumulative number of 118,446 ± 15,343 CFC/10^6 panned Lin⁻34⁺DR⁻ cells (n = 3) over 12 wk of culture (Fig. 3 *D*), which is similar to the cumulative number of colonies recovered from LTBMC initiated with plated Lin⁻34⁺DR⁻ cells (103,474 ± 2,432 CFC/10^6 plated Lin⁻34⁺ DR⁻ cells; n = 3; p = 0.5) (Fig. 3 *B*). Recovery of CFC from the adherent layers of LTBMC initiated with panned Lin⁻34⁺DR⁻ cells (Fig. 5 *D*) was identical to what we observed in LTBMC initiated with plated Lin⁻34⁺DR⁻ cells (Fig. 2 *D*). These results suggest that cells with LTBMC-initiating capacity in the Lin⁻34⁺DR⁻ fraction, but not from the Lin⁻34⁺DR⁺ fraction, have the capacity to adhere to irradiated stroma.

Culture of Cells Recovered from Panning Effluent in LTBMC. To confirm that LTBMC-initiating cells in the Lin⁻34⁺DR⁻ fraction are capable of adhering to established stroma, we plated the cells recovered in the panning effluent after panning with either Lin⁻34⁺DR⁺ or Lin⁻34⁺DR⁻ cells onto second stromal layers and analyzed both nonadherent and adherent fractions of such cultures for the presence of clonogenic cells during five consecutive weeks.

Nonadherent layers of LTBMC initiated with cells recovered

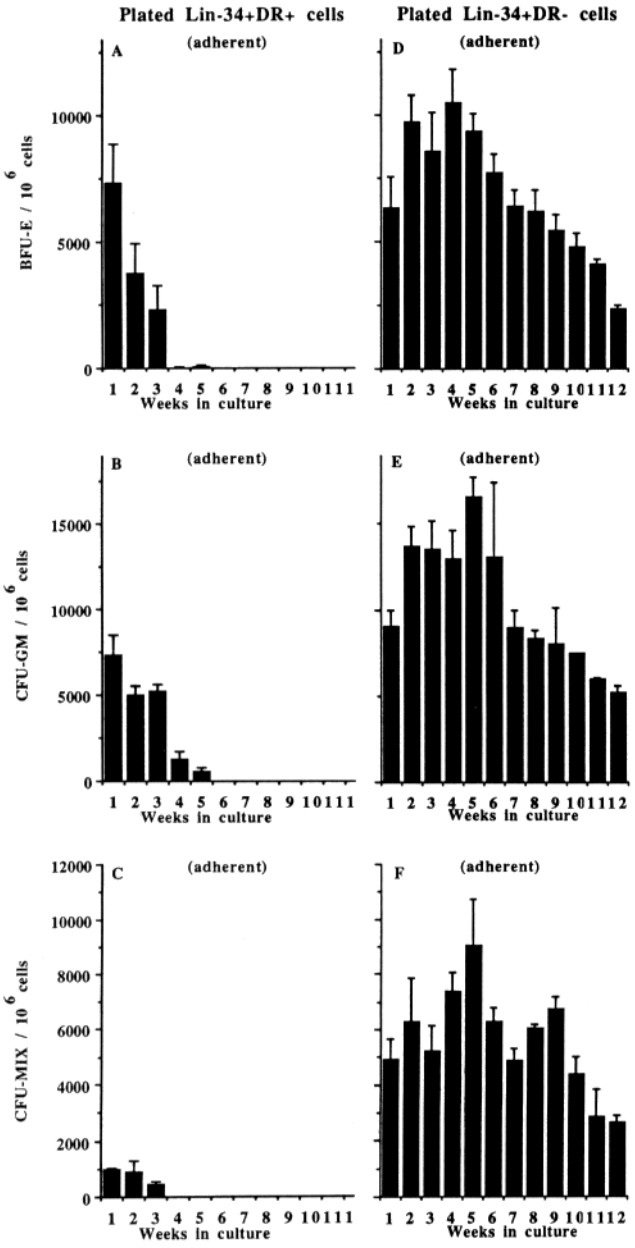

Figure 4. Lineage-negative BMMNC were separated by FACS according to low/very low horizontal and low vertical light scatter properties into Lin$^-$34$^+$DR$^+$ cells (*A–C*) and Lin$^-$34$^+$DR$^-$ cells (*D–F*). BFU-E (*A*), CFU-GM (*B*), and CFU-MIX (*C*) were recovered from adherent layers from LTBMC initiated with Lin$^-$34$^+$DR$^+$ plated cells. BFU-E (*D*), CFU-GM (*E*), and CFU-MIX (*F*) were recovered from adherent layers from LTBMC initiated with Lin$^-$34$^+$DR$^-$ plated cells. Data points are CFC recovered from weekly harvested adherent layers of LTBMC per 10^6 plated cells used to initiate the LTBMC. Weekly time points between weeks 1 and 3 represent mean ± SEM of seven experiments; data points at week 4–5 represent ± SEM of 10 experiments, data points between weeks 6 and 8 represent mean ± SEM of six experiments; data points between weeks 9 and 12 represent mean ± SEM of three experiments.

from panning effluent after panning with Lin$^-$34$^+$DR$^+$ cells contained high numbers of both single- and multi-lineage CFC (4,105 ± 836 CFC/10^6 cells used to initiate LTBMC with panned Lin$^-$34$^+$DR$^+$ cells) after 1 wk of culture in LTBMC (n = 6). There was a progressive decline in CFC recovered from LTBMC initiated with cells from the panning effluent of Lin$^-$34$^+$DR$^+$ cells over the following weeks, and only 25 ± 5 CFC/10^6 cells could be recovered from nonadherent layers of these LTBMC at week 5 of culture (n = 4). Similarly, high numbers of CFC were recovered during the first week of culture (7,680 ± 1,503 CFC/10^6 cells) (n = 6) from adherent layers of LTBMC initiated with cells from the panning effluent recovered after panning stroma with Lin$^-$34$^+$DR$^+$ cells, while a significant decline in CFC was observed over the following weeks. We recovered 750 ± 270 CFC cells at week 5 of culture (n = 4) (calculated for 10^6 Lin$^-$34$^+$DR$^+$ cells used to pan stroma). The number of CFC recovered in both nonadherent and adherent layers of LTBMC initiated with plated Lin$^-$34$^+$DR$^+$ cells and LTBMC initiated with cells recovered from the panning effluent after panning stroma with Lin$^-$34$^+$DR$^+$ cells was equivalent (p = NS). These data confirm that committed myeloid progenitors present in the Lin$^-$34$^+$DR$^+$ population are unable to adhere to irradiated BM-derived stroma when panned onto stroma for 2 h.

We also analyzed nonadherent and adherent layers of LTBMC initiated with cells recovered in the panning effluent recovered after panning stroma with Lin$^-$34$^+$DR$^-$ cells. At week 1 of culture, 1,077 ± 277 CFC were recovered from nonadherent layers and 2,006 ± 621 cells from adherent layers of LTBMC initiated with cells recovered in the panning effluent after stroma was panned with Lin$^-$34$^+$DR$^-$ cells (calculated for 10^6 panned Lin$^-$34$^+$DR$^-$ cells). Over the following weeks, we recovered a similar number of CFC from both nonadherent and adherent layers of such cultures. During the first 3 wk of culture, the number of CFC recovered from nonadherent layers of LTBMC initiated with cells recovered in the effluent after panning stroma with Lin$^-$34$^+$DR$^-$ cells was not statistically significantly different than that from LTBMC initiated with plated Lin$^-$34$^+$DR$^-$ cells, suggesting that more mature progenitor cells capable of generating CFC in the nonadherent layer of LTBMC in <3 wk do not adhere to irradiated stroma. At week 5 of culture, we recovered significantly less CFC from both nonadherent (2,116 ± 783 CFC; n = 4; $p \leq 0.001$) and adherent layers (3,407 ± 1,309; n = 4; $p \leq 0.001$) of LTBMC initiated with cells recovered in the panning effluent after panning stroma with Lin$^-$34$^+$DR$^-$ cells compared with LTBMC initiated with either plated Lin$^-$34$^+$DR$^-$ or panned Lin$^-$34$^+$DR$^-$ cells. This resulted in recovery of only 5,637 ± 2,142 LTBMC-initiating cells (Table 4) in such cultures, which is 11.9 ± 0.9% of the number of LTBMC-initiating cells recovered in LTBMC initiated with either plated or panned Lin$^-$34$^+$DR$^-$ cells. These data confirm that almost all LTBMC-initiating cells (88%) present in the lin$^-$34$^+$DR$^-$ population are capable of adhering to irradiated BM-derived stroma when panned for 2 h at 37°C in hydrocortisone-containing LTBMC media.

Discussion

In the present study, we have purified human primitive BM progenitor cells capable of initiating and sustaining he-

Table 3. *Recovery and Enrichment of LTBMC-initiating Cells*

BM populations	*n*	Percent cells	LTBMC-initiating cells (10^6 cells)	Enrichment	Percent recovery
BMMNC	10		122 ± 9.9		
$Lin^-34^+DR^+$	10	0.51 ± 0.09	721 ± 199	6.63 ± 2.4	3.3 ± 0.9
$Lin^-34^+DR^-$	10	0.20 ± 0.1*	46,866 ± 1,523*	424 ± 37*	68 ± 9.5*

LTBMC-initiating cells equals the sum of CFC recovered from nonadherent and adherent layers of LTBMC at week 5 of culture.
* $p \leq 0.001$.

matopoiesis in LTBMC. We demonstrate that the cells capable of adhering to allogeneic irradiated stroma, initiating hematopoiesis, and sustaining growth of committed progenitors in LTBMC are very small blast-like cells that are CD34 antigen positive but HLA-DR antigen negative. Furthermore, these primitive progenitors fail to express T and B lymphocyte or NK cell antigens and are CD11, CD15, CD71 antigen (lineage negative). These cells differ in several respects from cells that form single- or multi-lineage colonies upon direct culture in short-term methylcellulose progenitor assays. The latter are morphologically somewhat larger blasts, and these clonogenic cells, although $CD34^+$ and lineage negative, do express HLA-DR antigen on the cell surface. These observations are in accordance with recent reports that demonstrate that clonogenic cells are HLA-DR antigen positive, while more immature progenitor cells capable of forming blast cell colonies in semi-solid media (9) or on stromal layers (10, 11), or cells with LTBMC-initiating capacity (13), are HLA-DR antigen negative.

A two-step purification of low density BM cells by negative immunomagnetic selection and positive FACS enabled us to enrich the $Lin^-34^+DR^-$ cell fraction 420-fold compared with unmanipulated BMMNC obtained after Ficoll-Hypaque separation for primitive hematopoietic progenitor cells capable of initiating LTBMC. Our purification resulted in a two- to three-fold greater enrichment compared with other reports (12, 13), and is probably the result of the initial negative immunomagnetic selection step for CD2-, CD19-, and CD71-expressing progenitor cells. A significant number of cells with low vertical and horizontal light scatter properties that express CD34 antigen on the cell membrane coexpress CD71 (transferrin receptor) antigens. This fraction contains mainly erythroblasts, BFU-E and CFU-E progenitors, and to a lesser degree, CFU-GM progenitors (23, 24). Comparison of CFC present in BM subpopulations obtained after negative immunomagnetic selection with or without anti-CD71 antibody, followed by a positive selection using FACS for cells expressing CD34 and HLA-DR antigens, demonstrated a significantly lower recovery of CFC in both $34^+/DR^-$ and $34^+/DR^+$ fractions upon direct culture in methylcellulose assay when the initial depletion encompassed cells expressing the CD71 antigen. Others have demonstrated that additional depletion of $CD34^{3+}/HLA\text{-}DR^-$ progenitor cells from cells expressing CD71 antigens may increase the number of high proliferative potential colonies recovered in semi-solid cultures and increase significantly the proliferation capacity of this purified cell population in suspension cultures with combinations of early acting hematopoietic growth factors (25). Approximately 50% of the $CD34^+$ cells present in human BM coexpress the early B lymphocyte–associated CD19 and CD10 antigens (26–28), and a small fraction of $CD34^+$ BM cells stains positive for early T lymphocyte antigens (CD2, CD7) (29). Depletion of both $CD19^+$ and $CD2^+$ cells in the first immunomagnetic purification step may, therefore, has allowed us to enrich further for the cell fraction capable of initiating and maintaining hematopoiesis in LTBMC. Using this two-step purification method, we demonstrate here that the combination of positive selection for small blast-like cells that are CD34 antigen positive but HLA-DR antigen negative, combined with a more extensive negative selection depleting the population of $CD2^+$, $CD19^+$, and $CD71^+$ cells, results in a significant enrichment for the progenitor cells capable of initiating and sustaining long-term hematopoiesis in LTBMC (12, 13).

Using a previously described panning method (10), we demonstrate that progenitor cells with long-term in vitro repopulating capacity present in the $Lin^-34^+DR^-$ population have the capacity to adhere to pre-established irradiated stroma, while more committed clonogenic $Lin^-34^+DR^+$ cells do not have the ability to adhere to stromal layers when panned for a 2-h period in hydrocortisone containing LTBMC media. Secondary LTBMC cultures initiated with cells recovered from panning effluent after a 2-h panning of

Table 4. *Recovery of LTBMC-initiating Cells from Effluent Recovered after Panning Stroma with $Lin^-34^+DR^+$ or $Lin^-34^+DR^-$ Cells*

BM populations	Percent recovery of cells (n = 10) in panning effluent	Recovery of LTBMC-initiating cells (n = 4) per 10^6 cells
$Lin^-34^+DR^+$	79.1 ± 10.2	775 ± 278
$Lin^-34^+DR^-$	37 ± 2.05*	5,673 ± 2,141¶

* $p \leq 0.001$.
¶ $p \leq 0.05$.

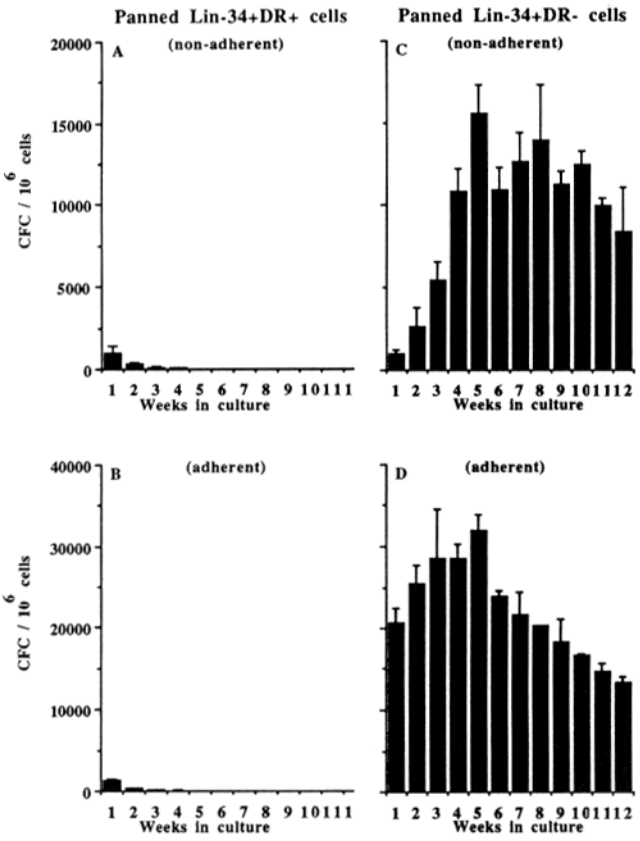

Figure 5. Lineage-negative BMMNC were separated by FACS according to low/very low horizontal and low vertical light scatter properties into Lin$^-$34$^+$DR$^+$ cells (*A* and *B*) and Lin$^-$34$^+$DR$^-$ cells (*C* and *D*). (*Top*) CFC recovered from nonadherent layers of LTBMC initiated with panned Lin$^-$34$^+$DR$^+$ cells (*A*) and panned Lin$^-$34$^+$DR$^-$ cells (*C*). (*Bottom*) CFC recovered from adherent layers of LTBMC initiated with panned Lin$^-$34$^+$DR$^+$ cells (*B*) and panned Lin$^-$34$^+$DR$^-$ cells (*D*). Data points are CFC recovered from weekly harvested nonadherent and adherent layers of LTBMC per 10^6 panned cells used to initiate the LTBMC. For nonadherent layers, data points between weeks 1 and 5 represent mean ± SEM of 10 experiments; data points between weeks 6 and 8 represent mean ± SEM of six experiments; data points between weeks 9 and 12 represent mean ± SEM of three experiments. For adherent layers, data points between weeks 1 and 3 represent mean ± SEM of seven experiments; data points at week 4–5 represent mean ± SEM of 10 experiments, data points between weeks 6 and 8 represent mean ± SEM of six experiments; data points between weeks 9 and 12 represent mean ± SEM of three experiments.

Lin$^-$34$^+$DR$^-$ cells onto stroma fail to generate significant numbers of LTBMC-initiating cells. The presence of small numbers of LTBMC-initiating cells in the panning effluent is probably due to incomplete adhesion of these primitive progenitor cells in one cycle of panning. Alternatively, subtle differences in adhesive properties of different immature progenitor cells may account for the presence of small numbers of LTBMC-initiating cells in the panning effluent recovered after panning stroma with Lin$^-$34$^+$DR$^-$ cells. The recovery of very small numbers of CFC from adherent and nonadherent layers of LTBMC initiated with panned Lin$^-$34$^+$DR$^+$ suggests that a small fraction of more mature progenitor cells may have the capacity to adhere to stromal elements. Alternatively, the recovery of CFC from LTBMC initiated with panned Lin$^-$34$^+$DR$^+$ cells may be due to the inadvertent incomplete removal of unbound cells during the panning procedure. These findings confirm and extend earlier observations that immature hematopoietic progenitor cells are capable of adhering to stroma (10, 16). Our studies provide the first evidence that these cells are present exclusively in the highly purified Lin$^-$34$^+$DR$^-$ cell fraction, and can initiate and sustain generation of committed myeloid progenitors in adherent and nonadherent layers of LTBMC for at least 3 mo.

Primitive progenitor cells in the Lin$^-$34$^+$DR$^-$ fraction lodge in the stromal layer and are capable of sustaining the production of multi- and single-lineage CFC, initially in the adherent layer only, but later also in the overlying supernatant, for at least 12 wk. We hypothesize that a small fraction of the Lin$^-$34$^+$DR$^-$ cells cultured in LTBMC lodge in the adherent layer of LTBMC and possess extensive self-renewal capacity and the capacity to generate mature clonogenic cells upon weekly feeding. Alternatively, most of the cells present in the Lin$^-$34$^+$DR$^-$ fraction do not possess self-renewal capacity but eventually differentiate into more mature clonogenic cells after variable periods of quiescence. In favor of our hypothesis are reports that a small number of cells present in either CD34$^+$ cells (18, 19) or CD34$^+$/HLA-DR$^-$ (9) cells are capable of generating blast-cell colonies with self-renewal capacity upon plating in semisolid culture systems. The kinetics of appearance and continued generation of CFC in both the nonadherent and adherent fractions of LTBMC with Lin$^-$34$^+$DR$^-$ cells also favor our hypothesis. We demonstrate an initial increase in CFC recovered from both fractions reaching a maximum at 5 wk after the start of the culture. This is followed by a progressive 40–50% decline over the next 7 wk of culture. The total number of colonies harvested weekly from these cultures (nonadherent and adherent layers) during this phase represents 2–3% of the total number of cells used to start the LTBMC. We speculate, therefore, that the continued generation of CFC after week 5 of LTBMC culture may be derived from a small fraction of the Lin$^-$34$^+$DR$^-$ cells with extensive self-renewal capacity. Since the total number of colonies recovered in the nonadherent and adherent layers of LTBMC initiated with Lin$^-$34$^+$DR$^-$ cells is 5–10-fold higher than what has been described in semi-solid culture systems (7, 8, 14), we speculate that the combination of growth stimulatory factors and micro-environment provided by the LTBMC may provide a superior environment for the support of immature progenitors than a semi-solid culture system containing combinations of hematopoietic growth factors. The initial twofold higher number of CFC recovered from nonadherent and adherent layers of LTBMC at week 5–7 may represent CFC derived from the same immature progenitor cells that give rise to the CFC recovered during the plateau phase of the cultures, which became progressively less active when the cultures age. Alternatively, a different progenitor cell with a less extensive self-renewing capacity may generate CFC for a shorter period of time.

In this LTBMC model, we used allogeneic, preestablished irradiated stromal layers. We do not know to what extent irradiation with 1,000 cGy is responsible for the selective adherence and the proliferation of the $Lin^-34^+DR^-$ cells in the pre-established stromal layers. Studies in mice demonstrate that irradiation of stromal layers induces increased production of both CSF for granulocyte, monocyte, and megakaryocyte colonies for up to 14 d after irradiation (30). Similarly, increased levels of CSF have been detected in the circulation of mice after whole body irradiation (31). The possibility exists that irradiation induces a concurrent increase in expression of recognition molecules for primitive hematopoietic progenitors. Gordon et al. (32) reported, however, that irradiation of stromal layers had no influence on the capacity of BM cells to adhere to stroma and to generate either committed colonies or blast-CFC.

In conclusion, the $Lin^-34^+DR^-$ fraction contains immature hematopoietic progenitor cells that specifically adhere to and proliferate in stromal layers. These primitive progenitors are capable of initiating and sustaining hematopoiesis in a LTBMC system for at least 3 mo. Further studies are needed to define more extensively the immunophenotype of the cell(s) responsible for this long-term hematopoiesis. Our in vitro culture system, in which highly purified primitive progenitors are panned onto pre-established irradiated stroma, may allow us to define further the importance of several putative homing receptors on marrow progenitors and their ligands in the BM-derived stromal layers (33–37).

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