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Identification and characterization of hematopoietic stem cells from the yolk sac of the early mouse embryo

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ABSTRACT **The yolk sac is the first site of hematopoiesis in the mammalian embryo. However, little is known about the initial stem cells in the yolk sac. We have isolated hematopoietic stem cells from early mouse embryonic yolk sac by using a sequential protocol of nonadherence to plastic, density gradient centrifugation, immunocytoadherence, and cell sorting. Isolated, nonadherent, density <1.077-g/cm^3, surface antigen AA4.1$^+$, wheat germ agglutinin bright (WGA^{bright}) cells give rise to multiple lineages, including T cells, B cells, and myeloid cells, as detected by using fetal thymus organ culture, S17 stromal feeder layers, or methylcellulose culture colony-forming cells, respectively. AA4.1$^+$, WGA^{bright} cells expressed high levels of heat-stable antigen (HSA) and CD45 (Ly-5) but did not significantly express major histocompatibility complex antigens, CD44, or Sca-1. Peak stem cell concentration is reached by day 11, before stem cells can be found in the liver, omentum, or thymus. *In vivo* long-term reconstitution of lethally irradiated mice was effected by as few as 720 AA4.1$^+$, WGA^{bright} yolk sac cells, but it required addition of a subset of bone marrow cells capable of providing immediate (short-term) radiation protection. Yolk sac donor-derived T cells, B cells, and macrophages were readily identified 6 months after transfer of yolk sac-derived stem cells. We suggest that, because of their cell surface phenotype as well as their capacity to differentiate *in vitro* and *in vivo*, the cells isolated from the mouse embryonic yolk sac may include the most primitive hematopoietic pluripotential stem cells yet identified.**

Embryonic stem cells can differentiate not only into hematopoietic cell lineages but also into all other lineages—i.e., they appear to be totipotent. The first time that commitment to hematopoiesis can be detected during mammalian embryogenesis is shortly after implantation, when blood islands develop in the extraembryonic yolk sac membrane (1, 2). Assignment to hematopoiesis and subsequent blood cell differentiation occur in the yolk sac even before the formation of the embryonic liver rudiment, before the formation of a thymic anlage, and well before the appearance of bone marrow, spleen, lymph nodes, or other hematopoietic organs.

The end products of hematopoietic differentiation include B lymphocytes, T lymphocytes, erythrocytes, granulocytes, megakaryocytes, and mast cells. Different lineages diverge from the original stem cell pool, become sensitive to and dependent on different growth factors, and manifest progressively more restricted developmental potential. Most of our knowledge concerning this lineage divergence has come from a careful analysis of adult bone marrow cells (3). However, because bone marrow cells are derived from cells already committed to hematopoietic differentiation they cannot provide us with information on prior events in the commitment process.

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In mammalian embryos the liver is the major hematopoietic organ beginning at the time of organogenesis—i.e., day 12+ in the mouse (4-7). Commitment to hematopoiesis may, however, occur before the liver is formed.

Our studies focus on the developmental potential of cells found in the embryonic yolk sac prior to the formation of any other site of hematopoiesis. Prior studies using mass yolk sac cultures have demonstrated that within the yolk sac are cells that can develop into several T-cell subsets (10–13), as well as immunoglobulin-producing cells, myeloid cells, and nucleated erythrocytes (14–16). Our goal has been to characterize the primitive hematopoietic stem cells, to study their colony-forming potential *in vitro* and *in vivo*, and to define the conditions under which clonal proliferation and differentiation of the various lineage precursors can occur.

MATERIALS AND METHODS

Mice. C57BL6/Au (Thy-1.2), C57BL6/Ka (Thy-1.1), and BALB/cAu were from our own colony. C57BL/6-Ly-5.2 congenic mice were initially obtained from Stanford University. Embryonic age was determined by observation of vaginal plugs, the morning of appearance being designated as day 0.

Cell Suspensions. Yolk sacs were dissected to eliminate maternal blood, mechanically disrupted, and filtered through 15-μm nylon mesh. Bone marrow was obtained from the long bones of adult mice.

Density Gradient Centrifugation. Yolk sac cells were separated by using discontinuous gradients of Percoll (Sigma) (1.054, 1.066, and 1.077 g/cm^3 in 0.15 M NaCl). Fractions were washed in PBS/30% fetal bovine serum (FBS) and resuspended in RPMI 1640 medium/30% FBS.

Removal of Adherent Cells. Gradient-separated yolk sac cells were incubated in tissue culture dishes at 37°C. Nonadherent cells were collected after 1 hr.

Cell Isolation by Immunocytoadherence. Polystyrene dishes (16 × 100 mm, Falcon 1001) were precoated with protein G-purified AA4.1, a rat IgG monoclonal antibody specific for the mouse cell surface antigen AA4.1 (3 ml, 10 μg/ml in 0.05 M Tris·HCl/0.15 M NaCl, pH 9.5, room temperature, 1 hr). After washing and blocking with PBS/1% FBS, cells were layered onto antibody-coated plates in 5 ml of Earle's balanced salt solution (EBSS)/1% FBS and incubated at 4°C for 1 hr. Nonadherent cells were removed, and plates were washed 8–10 times with PBS/1% FBS. The remaining adherent cells were then removed by forceful pipetting (cf. ref. 17).

Flow Cytometry. Cells were incubated with antibodies for 30 min, stained with second-step reagents for 30 min, then resuspended in PBS/1% bovine serum albumin. Flow cytometric analysis was carried out with a FACScan instrument (Becton Dickinson). Sorting was carried out with a Becton

Abbreviations: FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; PE, phycoerythrin; HSA, heat-stable antigen; IL, interleukin; WGA, wheat germ agglutinin; CM, conditioned medium; CFC, colony-forming cells; HPP-CFC, high proliferation potential CFC; CFC-GM, granulocyte–macrophage CFC.

Dickinson FACStar^{PLUS} instrument (488 nm, 100-mA current, 70-μm nozzle aperture).

Reagents. Reagents included fluorescein isothiocyanate (FITC)-labeled Thy-1.2, FITC-CD45 (Ly-5, T200), FITC-CD8, and phycoerythrin (PE)-labeled CD4 from Becton Dickinson; PE-Thy-1.2 and FITC-CD44 (Pgp-1) from PharMingen; Thy-1.1 (clone HO-22-1) and heat-stable antigen (HSA) from the American Type Culture Collection; AA4.1 antibody from J. P. McKearn (18); AL21 (anti-Ly-6C) from A. M. Stall (19); antibody 14.8 (anti-B220) from P. Kincade (Oklahoma Medical Research Institute); Sca-1 from I. L. Weissman (20); H-2K^{b}IA^b (alloantiserum no. 21-107-08-1) from the National Institutes of Health repository; and T-cell antibody JORO37.5 from R. Palacios (21). We received gifts of mouse interleukin (IL)-3 from J. Ihle (Frederick Cancer Research and Development Center, Frederick, MD); recombinant murine stem cell factor, IL-7, and granulocyte–macrophage colony-stimulating factor (GM-CSF) from Immunex; and additional GM-CSF from Promega. We purchased FITC-labeled wheat germ agglutinin (FITC-WGA) from Sigma, Tricolor from Caltag (South San Francisco, CA), and second-step reagents from various commercial sources.

Reconstitution of Stem Cell-Depleted Thymic Rudiments (Organ Culture). Protocols were those reported by Jenkinson *et al.* (22). Day 14 fetal thymic lobes were placed on Nuclepore filters (0.8 mm pore size) supported by Gelfoam sponges (Upjohn) in 35-mm Petri dishes (Falcon 3003) containing 1.5 ml of RPMI 1640/10% FBS, 1.35 mM deoxyguanosine (Sigma), and antibiotics. After culture for 5 days thymus lobes were washed, then cocultured with 10^3 to 10^5 unfractionated or 10^1 to 10^3 sorted cells for 1–2 days in hanging drop culture using an inverted Terasaki plate. The lobes were washed free of unincorporated cells, then placed in organ culture (Nuclepore on Gelfoam) for 12–20 days.

Methylcellulose Culture Assay of Colony-Forming Cells (CFC). A stock solution of methylcellulose was prepared as described (23). Phytohemagglutinin-conditioned medium (PHA-CM) was prepared by culturing 2×10^6 C57BL/6 spleen cells per ml in RPMI 1640/10% FBS with PHA (Sigma) at 2.5 μg/ml, 3% human serum, and 50 μM 2-mercaptoethanol for 3 days. L-929 conditioned medium (L-CM) was prepared by culturing L929 cells in Dulbecco's minimal essential medium (DMEM)/10% FBS for 7 days.

Yolk sac cells (10^4 to 10^5 for unsorted, or 10^2 to 10^3 for sorted) were seeded in 0.8% methylcellulose supplemented with 15% PHA-CM and 15% L-CM, cultured for 7 days, and scored for colony formation. Groups of >40 cells were considered a colony, and colonies with diameters > 0.5 mm (containing >5000 cells) were considered to be "high proliferating potential" colonies (24). Groups of cells containing no more than 40 cells were considered to be clusters. Colonies were picked, applied to slides, and stained with DiffQuik (Baxter).

Stromal Cell Coculture Assay for B-Cell Differentiation. S17 bone marrow-derived stromal cells provided by Kenneth Dorshkind (University of California, Riverside) were grown to confluency in six-well Falcon culture plates. The medium for B-cell precursor differentiation consisted of RPMI 1640 with 10% FBS, 100 μM 2-mercaptoethanol, and recombinant human IL-7 at 1000 units/ml (25).

To determine the number of B-cell precursors, 2000 irradiated (2000 rad; 1 rad = 0.01 Gy) S17 cells were placed in each well of 96-well culture plates. After 24 hr, test cells were added and cultured for 2 weeks. Calculations of precursor frequency were made as described in the legend of Fig. 1.

Preparation of Bone Marrow Cell Fractions Depleted of Cells with Long-Term Reconstituting Ability. Counterflow centrifugal elutriation (CCE) was used to separate cell fractions depleted of cells with long-term reconstituting ability as described by Jones *et al.* (26). Cells were loaded ($\approx 2 \times 10^8$) at a flow rate of 15 ml/min and a rotor speed corresponding to $1260 \times g$. Cells were eluted by changing flow rates, 300 ml as a fraction at each flow rate. After 25 ml/min, 29 ml/min, and 33 ml/min, cells remaining in the chamber were collected as the Rotor-Off fraction and used as cells depleted of long-term reconstituting ability.

Irradiation and Reconstitution of Adult Mice. Two- to 3-month-old male C57BL/6-Ly-5.2 mice were lethally irradiated (cesium, 1130 rad, split dose >3-hr intervals, 166 rad/min.) Isolated yolk sac cells (720) from C57BL/6Au (Ly-5.1) mice along with 2×10^4 long-term reconstituting cell-depleted bone marrow cells from C57BL/6-Ly-5.2 mice were injected i.v. into irradiated congenic hosts.

RESULTS

Stem Cell Frequency Based on Density Centrifugation. The number of precursors for myeloid lineage found in methylcellulose cultures peaked at day 11 (Fig. 1*A*). The number of precursors capable of developing into T cells in stem cell-depleted thymus rudiments also peaked at day 11 (Fig. 1*B*).

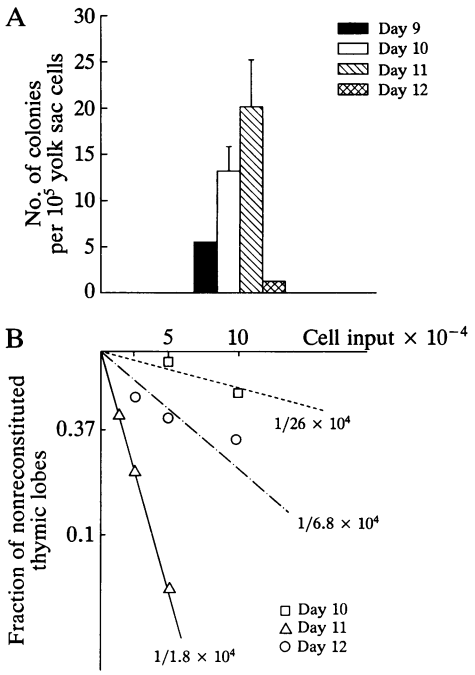

FIG. 1. Precursor frequency of myeloid and T cells in unseparated yolk sac cells. Yolk sac cells were dissected at day 9 to day 12 of gestation. (*A*) Cells were assayed in methylcellulose cultures supplemented with CM for 7 days. The colonies were counted under an inverted microscope. (*B*) Yolk sac cells were added to stem cell-depleted 14-day embryonic thymus cultures. The number of precursor cells was calculated by limiting dilution analysis. The number of stem cells capable of reconstituting fetal thymus lobes in organ culture was calculated on the basis of the Poisson zero-order term distribution formula $F_0 = e^{-u}$ or $-\ln F_0 = u$, where F_0 represents the probability F that a culture contains 0 stem cells, e is the base of natural logarithms, and u is the average number of stem cells per thymus. The responding and nonresponding cultures were distinguished by checking the presence of donor-derived T cells by flow cytometric analysis of allele-specific Thy-1. For each experiment the fraction of nonresponding cultures was plotted on a logarithmic scale against the cell dose seeded into the stem cell-depleted thymus. From the formula it can be calculated that the dose which on average contains one stem cell ($u = 1$) is the x value determined from this plot corresponding to 37% nonresponding culture (e.g., 1 stem cell per 1.8×10^4 input cells on day 11). The goodness-of-fit of the data was tested by using the correlation coefficient, while the line was fitted by the least-squares method (27).

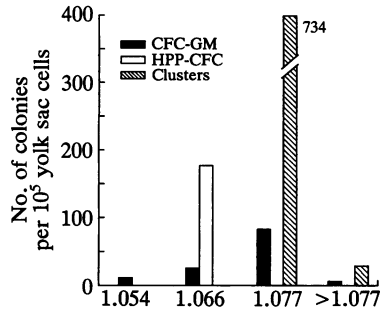

FIG. 2. Density separation of yolk sac cells. Day 11 yolk sac cells were separated by using discontinuous Percoll gradient centrifugation. Fractions of density 1.054, 1.066, 1.077, and >1.077 g/cm³ were collected and washed, then seeded in methylcellulose in CM for 1 or 2 weeks.

Eleven-day yolk sac cells were separated into density fractions of 1.054, 1.066, 1.077, and >1.077 g/cm³ and assayed in methylcellulose. As shown in Fig. 2, high proliferation potential colony-forming cells (HPP-CFC), believed to be more primitive precursors of granulocyte–macrophage CFC (CFC-GM), were most enriched in the 1.066-g/cm³ fraction (176 colonies per 10^5 cells). These colonies were not seen in unseparated fractions. CFC-GM (83 colonies per 10^5 cells) as well as clusters were found to be most enriched in the 1.077-g/cm³ fraction. By day 14, however, colonies derived from the 1.077- and the >1.077-g/cm³ fractions degenerated, and the HPP-CFC derived from the 1.066-g/cm³ fraction had spread out to form an adherent monolayer. In contrast, a low but significant number ($12/10^5$) of HPP-CFC now appeared in the 1.054-g/cm³ fraction, suggesting that these cells were more primitive and had to undergo some differentiation before becoming detectable as CFC.

Isolation of Yolk Sac Hematopoietic Stem Cells by Use of Plastic Adherence Properties, Density Centrifugation, Immunocytoadherence, and Cell Sorting. Day 11 yolk sac cells with density <1.077 g/cm³ were dispensed into plastic culture dishes to separate adherent (60–70%) from nonadherent (30–40%) cells. The nonadherent cells were then plated into AA4.1 antibody-coated plastic dishes to yield ≈0.1% $AA4.1^+$ cells by immunocytoadherence. These three steps enriched the stem cell population about 320-fold (Fig. 3). In contrast,

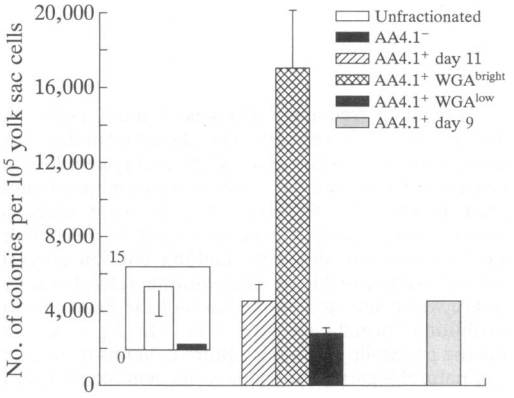

FIG. 3. Myeloid precursors in $AA4.1^+$ WGA^{bright} yolk sac cells. The density <1.077 g/cm³ yolk sac cells were collected from Percoll gradients. After removal of cells adherent to plastic, $AA4.1^+$ cells were isolated by immunocytoadherence to AA4.1 antibody-coated Petri dishes. Those that did not bind to anti-AA4.1 were harvested as $AA4.1^-$ cells and those that adhered were collected as $AA4.1^+$ cells. Using a FACStar cell sorter, we then further fractionated the $AA4.1^+$ cells into WGA^{bright} and WGA^{low} populations based on FITC-WGA binding. Each fraction was cultured for 7 days either in CM or with added growth factors. Error bars represent SD.

the $AA4.1^-$ cells contained <3% CFC, possibly reflecting contamination by $AA4.1^+$ cells. Finally, the enriched $AA4.1^+$ cell suspension was labeled with FITC-WGA, and the WGA^{bright} cells (20–30%) were isolated by flow cytometry/cell sorting (cf. ref. 28). The size of cells was intermediate between the sizes of lymphocytes and macrophages, and the cells showed the typical uniform staining pattern of hematopoietic stem cells (29).

Identification of Myeloid Precursors. The isolated plastic-nonadherent, density <1.077-g/cm³, $AA4.1^+$, WGA^{bright} cells in methylcellulose culture gave rise to macrophages, monocytes, megakaryocytes, blast cells, and granulocytes. A >1200-fold enrichment was achieved for myeloid progenitor cells—i.e., 1/5 to 1/10 cells were able to form myeloid colonies in methylcellulose cultures (Fig. 3). Secondary colonies could be generated from blast colonies after 7 days *in vitro*, providing evidence for the primitive nature of these cells (data not shown). To exclude fetal liver as a potential source of these cells (30) we studied day 9 yolk sac cells—i.e., prior to the appearance of the liver rudiment. The number of colony-forming $AA4.1^+$ cells from day 9 yolk sac was similar to that of day 11 (Fig. 3) yolk sac.

Identification of T-Lymphocyte Precursors. The isolated cells were seeded into stem cell-depleted thymus rudiments, using 20 to 280 yolk sac cells/lobe. After 12–14 days of culture, donor-derived T cells were identified on the basis of Thy-1.1/1.2 allelic differences. Three-color analysis showed donor-derived (Thy-1.2) T cells, including double-positive ($CD4^+$ $CD8^+$), double-negative ($CD4^-$ $CD8^-$), and single-positive ($CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$) cells (Fig. 4*A*). One of 39–88 cells was able to reconstitute an empty thymic lobe, compared with 1 of 3.7×10^4 (average from three separate experiments) in unseparated day 11 yolk sac, an achievement of 1480-fold enrichment (Fig. 4*B*).

Although care was always taken to avoid contamination by maternal blood, one experiment was carried out using an F_1

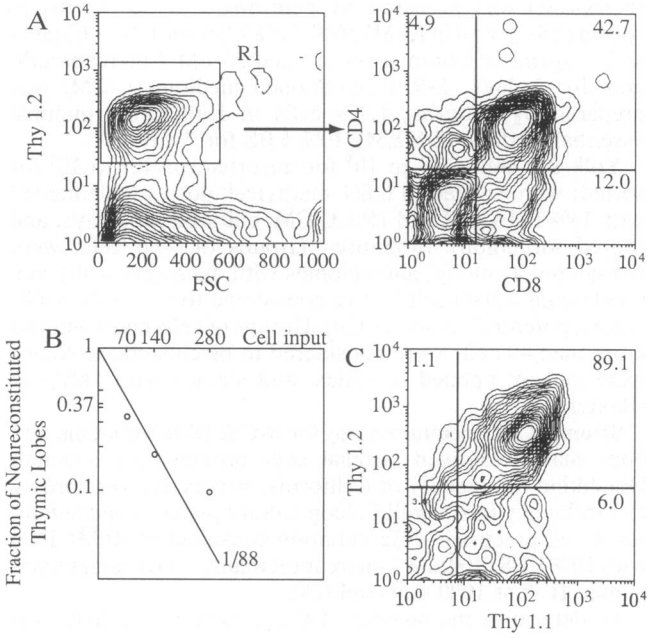

FIG. 4. T-lymphocyte precursors in $AA4.1^+$, WGA^{bright} yolk sac. (*A*) Three-color analysis of donor-derived T cells cultured in fetal thymus organ cultures. Contour plot shows CD4 and CD8 expression of cells gated on Thy-1.2 expression. Percentages of cells are given in the corners of the quadrants. (*B*) Limiting dilution analysis of T-cell precursor frequency in $AA4.1^+$, WGA^{bright} yolk sac cells. (*C*) (Thy-1.2 × Thy-1.1)F_1 yolk sac cells were distinguished on the basis of Thy-1.1 and Thy-1.2 expression to exclude the possibility of contamination by $Thy\text{-}1.2^+$ maternal blood cells.

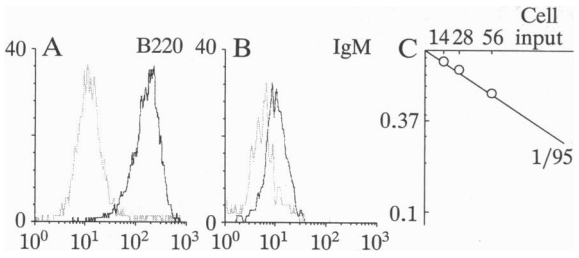

FIG. 5. B-lymphocyte precursors in AA4.1$^+$, WGA^{bright} yolk sac cells. AA4.1$^+$, WGA^{bright} cells were plated onto S17 bone marrow stromal cell feeder layers, and the medium was supplemented with IL-7 at 1000 units/ml for 14 days. (*A*) Nonadherent cells labeled with 14.8 (anti-B220) antibody. The dotted line represents isotype control. (*B*) Nonadherent cells labeled with goat antibodies to mouse IgM. (*C*) Limiting dilution analysis of B cell precursors in AA4.1$^+$, WGA^{bright} yolk sac cells.

[C57BL6/Au (Thy-1.2) × C57BL/Ka (Thy-1.1)] cross to generate Thy-1.1/1.2 hybrid embryos, then reconstituting Thy-1.1 stem cell-depleted thymic rudiments with the day 11 yolk sac cells. In this combination, cells of maternal origin would express Thy-1.2 but not Thy-1.1, while embryo-derived cells would express both Thy-1.1 and Thy-1.2. As shown in Fig. 4*C*, most Thy-1$^+$ cells expressed both alleles; a small percentage expressed only Thy-1.1, which survived from deoxyguanosine treatment; and none of them expressed only Thy-1.2.

Identification of B-Lymphocyte Precursors. The isolated putative stem cells were added to S17 monolayers and grown in the presence of IL-7. After 2 weeks, a significant number of B cells expressing B220 and surface-associated IgM were observed in these cultures (Fig. 5 *A* and *B*). Limiting dilution studies determined a precursor frequency of 1/95 in the AA4.1$^+$, WGA^{bright} population (Fig. 5*C*).

Expression of Other Phenotypic Cell Surface Markers on Isolated Yolk Sac Stem Cells. The stem cell antigen Sca-1 (Ly-6 A/E) was not expressed significantly on isolated hematopoietic stem cells (Fig. 6*A*), contrasting with the presence of this antigen on fetal-liver derived cells (Fig. 6*B*). We were also unable to detect expression of CD44 (Pgp-1), H-2K^{b}IA^b, or CD4 (Fig. 6*A*), JORO 37.5 (an antigen expressed on pro-T cells), or AL21 (Ly-6 C1/2) (not shown) on freshly isolated purified stem cells. Sorting on the basis of the highest-intensity Sca-1 or Pgp-1 cells did not enrich stem cells for any lineage. However, HSA and CD45 (Ly-5) were expressed on the AA4.1$^+$ hematopoietic stem cells.

Identification of Long-Term Bone Marrow Reconstituting Stem Cells. Transfer of 720 plastic-nonadherent, density <1.077-g/cm^3, AA4.1$^+$, WGA^{bright} cells along with 2 × 10^4 bone marrow cells depleted of cells with long-term reconstituting ability (Rotor-Off fraction) by density elutriation achieved reconstitution of T cells, macrophages, and granulocytes in irradiated adult congenic mice (Table 1). No significant numbers of donor-derived cells were detectable 15 days after transfer. Donor T cells were readily detected by 2 months after transfer, and made up more than 50% of T cells at 4 and 6 months. The 720 plastic-nonadherent, density <1.077-g/cm^3, AA4.1$^+$, WGA^{bright} cells alone did not provide radiation protection. As expected, the Rotor-Off fraction of bone marrow cells did not show significant reconstitution 2 months after transfer into irradiated mice (cf. ref. 26).

DISCUSSION

Our study shows that the early murine yolk sac contains hematopoietic stem cells capable of differentiating into B cells, T cells, and myeloid cells prior to the presence of such cells in the developing embryonic liver, before the formation of the thymic rudiments, and well before bone marrow makes its appearance during ontogeny.

Although there is as yet no evidence that the yolk sac stem cells we have identified migrate into fetal liver and thymus, our results are consistent with that view. The surface phenotype is clearly more primitive, for while they share with fetal liver stem cells the expression of AA4.1 and CD45, they label more strongly than fetal liver cells with antibodies to HSA, an antigen whose expression decreases during hematopoietic differentiation. They do not yet express significant levels of major histocompatibility complex antigens, CD44, JORO 37.5, Sca-1, or Ly-6C, all of which are found in fetal liver stem cells.

There were high numbers of cells capable of myeloid differentiation (1/5 to 1/10) compared with cells that could develop into T cells in stem cell-depleted thymus rudiments (1/44 to 1/88) or into B cells (1/95) in coculture with S17 bone marrow stromal cell monolayer. Possible reasons for this difference include: (*i*) many AA4.1$^+$, WGA^{bright} cells may already have become committed to the myeloid lineage; (*ii*) some step in prethymic differentiation [e.g., expression of CD44 or major histocompatibility complex antigens (32, 33)] must occur before thymic seeding can be achieved; (*iii*) culture conditions are not ideal (e.g., poor contact of yolk sac cells with the thymic rudiment; residual deoxyguanosine; deficient growth factors for B-cell differentiation); and (*iv*) successful B-cell/S17 cell interactions may also require a prior step in differentiation (e.g., expression of adhesion molecules). Moreover, some cells growing on S17 monolayers express cytoplasmic μ chain immunoglobulin without detectable surface IgM expression, indicating that the number of clonal B-cell precursors was greater than detected (34).

The fact that as few as 720 AA4.1$^+$, WGA^{bright} yolk sac cells give rise to a high percentage of donor-derived T cells, B cells, and macrophages after 6 months indicates that the yolk sac may contain a preponderance of primitive multipotential stem cells. Unlike the fetal liver or bone marrow, the yolk sac contains few cells capable of forming macroscopic spleen colonies (CFU-S) in irradiated mice. Recent experiments have shown that CFU-S represent stem cells that possess short-term reconstituting ability distinct from stem cells capable of long-term engraftment (8, 9). Zipori (35) suggests that the ability of a cell to proliferate rapidly to form

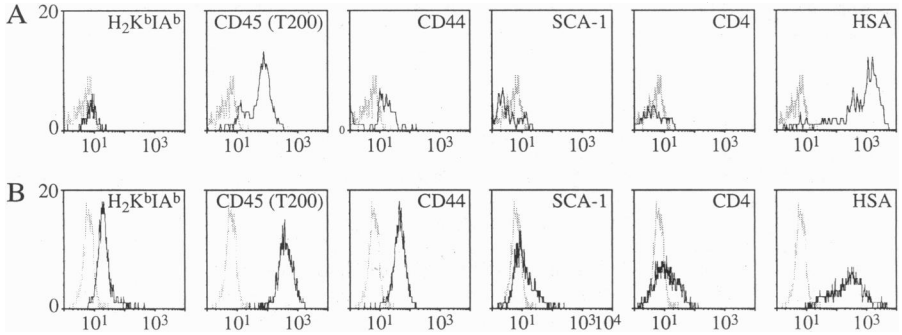

FIG. 6. Comparisons of cell surface phenotype of AA4.1$^+$ yolk sac and fetal liver cells. AA4.1$^+$ cells were stained with antibodies to H-2K^{b}Ia^b, CD45, CD44 (Pgp-1), Sca-1, CD4, and HSA. The dotted lines represent isotype controls. (*A*) Day 11 yolk sac. (*B*) Day 14 fetal liver.

Table 1. Long-term reconstitution of lethally irradiated mice by yolk sac stem cells

Cells	No. of cells	Surviving/ total	Time after reconstitution, months	Tissue analyzed	No. of animals reconstituted/ total	Percentage of cells in reconstituted animal derived from donor		
						T cells	Macrophages	Granulocytes
Unseparated YS	2×10^6	11/23	2	Thymus	1/8	99		
Rotor-Off BM	2×10^4	19/29	2	Thymus	0/10			
Unseparated YS + Rotor-Off BM	2×10^6 + 2×10^4	22/29	2	Thymus	5/8	65.2 ± 8.7		
AA4.1$^+$, WGA^{bright}	720	0/9						
AA4.1$^+$, WGA^{bright} + Rotor-Off BM	720 + 2×10^4	8/24	15 days	PBL	0/5	0.6 ± 0.4*		
			2	PBL	8/8	20.5 ± 2.3	6.9 ± 3.6	
			4	PBL	7/7	65.7 ± 7.7	58.7 ± 5.5	31.3 ± 8.4
			6	Spleen	7/9	55.0 ± 14.7	61.5 ± 9.6	67.7 ± 8.7

For transplantation experiments using unseparated yolk sac (YS) cells, cells prepared from C57BL/6ka (Thy-1.1) were transplanted into ^{137}Cs γ-irradiated C57BL/6 (Thy-1.2) congenic mice alone or along with the Rotor-Off fraction of bone marrow (BM) cells prepared from C57BL/6 (Thy-1.2) mice. In these experiments only thymocytes were assayed for the presence of Thy-1.1$^+$ cells. For transplantation experiments using sorted cells, nonplastic-adherent, density <1.077-g/cm^3, AA4.1$^+$, WGA^{bright} cells were isolated from C57BL/6-Ly-5.1 mice and transplanted into ^{137}Cs γ-irradiated C57BL/6-Ly-5.2 congenic hosts alone or along with Rotor-Off fraction prepared from C57BL/6-Ly-5.2 mice. At the times indicated above, mice were examined for presence of Ly-5.1$^+$ Thy-1.2$^+$, Ly-5.1$^+$ Mac1$^+$, and Ly-5.1$^+$ antigranulocyte$^+$ cells. PBL, peripheral blood leukocytes.

*Ly-5.1 marker was used to identify donor cells.

a macroscopic clone is inversely related to its ability to engraft, which is the ultimate assay for a genuine stem cell. The AA4.1$^+$, WGA^{bright} cell fraction of the yolk sac includes a high proportion of these genuine stem cells.

Aside from being intrinsically interesting for identifying the earliest hematopoietic stem cells, these findings also have practical applications. For example, graft rejection and graft-versus-host reactions are significant barriers to successful bone marrow engraftment, problems which are not fully circumvented by fetal liver cells. Yolk sac stem cells are more primitive, lack expression of major histocompatibility complex antigens, and have a greater reproductive potential than cells obtained from fetal liver or adult bone marrow (2).

Clearly, many questions remain to be answered: Can a single yolk sac stem cell give rise to all leukocyte phenotypes or is some restriction imposed at an even earlier time? Does the commitment to erythropoiesis occur prior to the expression of the AA4.1$^+$, WGA^{bright} phenotype? Are there multiple sites to which yolk sac cells migrate, and if so is there a selective distribution of cells related to their expression of cell adhesion/recognition molecules? Our identification and isolation of yolk sac stem cells are important first steps towards obtaining answers to these questions.

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