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At day 8–8.5 of mouse development the yolk sac, not the embryo proper, has lymphoid precursor potential *in vivo* and *in vitro*

(lymphocyte development/stromal cells/thymic epithelium/hemopoiesis/stem cells)

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ABSTRACT **We have studied both *in vitro* and *in vivo* the formation of lymphocyte progenitors before blood circulation (day 9 of gestation) has started in the mouse embryo, and we have determined the tissue where this occurs. The results demonstrate that the yolk sac of embryos at day 8 and day 8.5 of gestation contains precursor cells that can give rise, *in vivo* and *in vitro*, to mature T and B lymphocytes. No lymphoid precursors were found in the embryo proper at this stage of mouse development. The yolk sac cells with lymphocyte precursor potential are most likely multipotent stem cells rather than cell-lineage-determined T- and/or B-lymphocyte progenitors. The defined *in vitro* assays described here that support differentiation of yolk sac stem cells along the T- or B-lymphocyte pathways also may now facilitate the study of the molecular events leading to cell-lineage commitment of lymphocyte progenitors in the mouse embryo.**

Hematolymphopoiesis occurs in distinct tissues/organs as the mouse embryo develops (1–4). The yolk sac appears at day 7 of gestation, the liver primordium at days 8–9, the thymic anlage at day 9, and the spleen and bone marrow at days 15–16. The heart starts beating at day 9 and blood circulation commences in the embryo (1).

The yolk sac is the first site where precursors of the erythroid cell lineage have been demonstrated (2, 5). Whether precursor cells of lymphocytes appear first in the yolk sac or in the embryo proper has been a matter of controversy over the years. Thus, different research groups reported that cells with precursor potential for T and/or B lymphocytes appear first in the yolk sac (5), in both yolk sac and embryo proper (6), or first in the embryo proper and later in the yolk sac (7, 8). A critical aspect in addressing this question is that the age of the embryos ought to be precisely defined, since material from embryos before day 9 of gestation is required. As blood circulation starts at day 9, it renders it nearly impossible to ascertain whether the lymphoid precursor cells found in a particular tissue at this time of development (or later) are actually formed there or have simply migrated there.

We have carried out experiments in which the presence of cells with lymphocyte precursor potential in the yolk sac and the embryo proper was studied both *in vivo* and *in vitro*. The age of each embryo was determined by counting the number of somite pairs. We used only material from embryos at day 8 and day 8.5 of gestation. The results of such experiments are the subject of the present communication.

MATERIALS AND METHODS

Animals. Hormone-treated C57BL/6 mice (gonadotropin, 5 units of Folligon 48 hr before mating, 5 units of Chorulon immediately before mating) were mated. The appearance of the vaginal plug was designated as day 0. Precise embryonic stages were determined by counting the pairs of somites under a dissection microscope equipped with a transilluminator. Embryos with 3–6 pairs of somites (approximately Carnegie stage 9) were considered as day 8 of gestation. The yolk sac of these embryos had no visible blood islands. Embryos with 7–12 pairs of somites (approximately Carnegie stage 10) were considered as day 8.5 of gestation and they had readily visible blood islands in their yolk sac. Cell suspensions from pooled whole conceptuses (embryo + yolk sac), embryo proper (embryos from which yolk sac was completely removed), and yolk sac were prepared as described by Wong *et al.* (9). Cells were washed and resuspended in culture medium or appropriate buffers as required and were used for fluorescence-activated cell sorter (FACS) analysis, PCR analysis, or injection into severe combined immunodeficiency (SCID) mice or to test T- and/or B-lymphocyte precursor potential in the *in vitro* systems described below.

Assay for Lymphoid Precursor Potential *in Vivo*. Cell preparations from whole conceptuses, embryo proper, and yolk sac (pool of 8–10 embryos per group) of day 8.5 C57BL/6 mouse embryos were directly injected into liver, spleen, and peritoneal cavity of sublethally irradiated [300 rads (3 Gy) of γ rays 3–6 hr before transfer of the cells] CB17 SCID mice (8- to 14-week-old male and female mice which had no detectable serum IgM and which were bred and maintained in the pathogen-free animal barrier facility in our institute) by a surgical procedure described before (10). SCID mice which were similarly treated but received no cells were included as negative controls. Sixteen weeks later, spleen mononuclear cell suspensions free of erythrocytes were prepared by standard procedures. The presence of donor-derived H-2b$^+$ T lymphocytes [T-cell receptor (TCR)/CD3$^+$, CD4$^+$, CD8$^+$] and H-2b$^+$ B lymphocytes (B-220$^+$, IgM$^+$) was determined by two-color FACS analysis (10–12) using the following antibodies (FITC, fluorescein isothiocyanate): biotin-H-2K^b (AF6-88.5), FITC-CD3 (145-2C11), FITC-CD4 (GK1.5), FITC-CD8 (53-6-72), FITC-B-220 (RA3-6B2), and FITC-IgM. Cells exposed to rat IgG and FITC or phycoerythrin (PE)-streptavidin were negative controls. Spleen cells from normal young adult C57BL/6 mice were used as a positive control for staining. Viable "lymphoid" cells were gated by forward and side scatters. Fluorescence emitted by single viable cells was measured with logarithmic amplification. The data obtained from 2.0×10^4 cells were analyzed with CONSORT 30 software.

T-Cell Differentiation *in Vitro*. Cell preparations from yolk sac ($2.0–5.0 \times 10^4$ cells per well) or from embryo proper

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Abbreviations: FACS, fluorescence-activated cell sorter; TCR, T-cell receptor; FITC, fluorescein isothiocyanate; PE, phycoerythrin; rIL3, rIL6, and rIL7, recombinant interleukins 3, 6, and 7; LPS, lipopolysaccharide; V, D, and J, variable, diversity, and joining.

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(lacking yolk sac, ≈3–4 embryos per well) were cultured on monolayers of the EH6 subcapsular thymic epithelial clone (10) on six-well Costar plates in the presence of recombinant interleukin (rIL) 3 (25–50 units/ml) (12), rIL7 (500 units/ml) (12), and F [final concentration 10%; supernatant from FLS4.1 stromal cells (12)] in a final volume of 1.5 ml per well of culture medium [Iscove's modified Dulbecco's medium + 2 mM L-glutamine + 50 μM 2-mercaptoethanol + gentamycin at 50 μg/ml and 7.5% fetal calf serum] at 37°C for 8–10 days. At day 5–6 of culture 1 ml of fresh culture medium was added to each well. The cells were harvested and washed and a portion of the cells were used for FACS analysis and DNA-based PCR analysis. Cells from the first culture (Fig. 1, bar A) (5×10^4 cells per well) were cultured on monolayers of the ET cortical thymic epithelial clone (10) in six-well Costar plates in a final volume of 1.5 ml of culture medium per well at 37°C for 8 days. The cells were harvested, washed, and used for FACS analysis.

B-Cell Differentiation *in Vitro*. Cell preparations from yolk sac ($2.0–5.0 \times 10^4$ cells per well) or from the embryo proper (lacking yolk sac, ≈3 embryos per well) were cultured on monolayers of the FLS4.1 fetal liver stromal cell line (12) (≈25% confluency) in six-well Costar plates in the presence of rIL3 (20 units/ml) (12), rIL6 (10–50 units/ml) (12), and rIL7 (500 units/ml) (12), in a final volume of 1.5 ml of culture medium (see above) at 37°C for 8–10 days. One milliliter of fresh culture medium containing rIL7 was added to each well at day 5–6 of culture. The cells were harvested and washed, and a portion of them were used in the second-step culture and the rest were used for FACS analysis and DNA-based PCR analysis. The cells from the first culture (Fig. 1, bar C) (5×10^4 cells per well) were cultured with the RP.0.10 bone marrow stromal clone (12) (≈25% confluency), rIL7 (500 units/ml), and *Escherichia coli* lipopolysaccharide (LPS; 40 μg/ml) in a final volume of 2 ml of culture medium at 37°C for 6–8 days. The cells were harvested, washed, and used for FACS and DNA-based PCR analysis.

FACS Analysis. Immunofluorescence staining and flow fluorocytometry (FACS) were carried out as described before (10–12), using a FACScan instrument (Becton Dickinson). All stainings were done in the presence of purified rat IgG (250 μg/ml) and heat-inactivated hamster serum (15%) to prevent nonspecific Fc-receptor binding of labeled antibodies. Viable cells were identified by using propidium iodide. The following antibodies specific for surface markers were used: biotin-PgP-1 (I42/5), biotin-c-kit receptor (ACK2), biotin-Joro 75, biotin-Joro 37-5, biotin-Thy1.2 (30H12), biotin-B-220 (14.8), FITC-TCR αβ (H57-597), FITC-TCR γδ (GL3), FITC-IgM. Second-step reagents were FITC-streptavidin (Vector Laboratories) or PE-streptavidin (Southern Biotechnology Associates, Birmingham, AL). Cells exposed to rat IgG and second-step reagents were negative controls. Mononuclear cells from day 14–15 fetal thymus and liver (for Joro 75, Joro 37-5, TCR γδ, c-kit), adult bone marrow (for PgP-1, B-220) and spleen (for Thy1.2, B-220, TCR αβ, IgM) were used as positive controls. Fluorescence emitted by single viable cells was measured with logarithmic amplification. Data collected from 10^4 to 2×10^4 cells were analyzed with CONSORT 30 software.

PCR Assays. DNA-based PCR was carried out with cell lysates obtained as described (13, 14) from cell samples of uninduced and induced cells from yolk sac or embryo proper and 15-day fetal thymocytes, adult thymocytes, or bone marrow mononuclear cells (for positive controls). Amplifications were made with the GeneAmp DNA amplification kit of Perkin–Elmer/Cetus, following the supplier's recommendations with the exception that 1.5–2.5 mM $MgCl_2$, bovine serum albumin at 100 μg/ml, 50–200 μM dNTPs, and 1.5–2.5 units of *Taq* DNA polymerase (Cetus) were used as required in 50-μl PCR mixtures which contained ≈3–5 μl of templates (≈3×10^4 to 5×10^4 genomes). The primers (Vδ1, Vδ4, Vδ6, Jδ1, Vγ1, Vγ5, Vγ6, Vγ7, Jγ1, 5′Dβ2, 5′Dβ1, 3′Jβ2, DHL, VO52, VH7183, VH558, JH3, Vκ, Jκ2, 5′ actin, and 3′ actin) and the DNA probes used and the cycles performed in the thermal cycler (Cetus) have been described in detail elsewhere (15). The PCR products were fractioned in agarose gels, blotted to nitrocellulose filters, and hybridized with ^{32}P-labeled appropriate probes, followed by autoradiography. Exposure times were usually between 0.5 and 2 hr.

RESULTS AND DISCUSSION

C57BL/6 mouse embryos at day 8 (3–6 pairs of somites) and at day 8.5 (7–12 pairs of somites) were obtained and the yolk sac was carefully dissected away. Only embryos in which the yolk sac could be completely removed were used, and we shall refer to them as embryo proper.

***In Vitro* Differentiation Along the T-Lymphocyte Pathway.** The presence of precursor cells to T-lymphocyte lineage in the yolk sac and in the embryo proper was determined in a two-step induction assay (Fig. 1, bars A and B). The putative precursor cells were first cultured on monolayers of the subcapsular thymic epithelial clone EH6 in the presence of exogenous rIL3, rIL7, and F [supernatant from the fetal liver stromal line FLS4.1 (12)] at 37°C for 10 days (Fig. 1, bar A). The hemopoietic cells were harvested and a portion of them were cultured on monolayers of the cortical thymic epithelial clone ET at 37°C for 8 days (Fig. 1, bar B). At the end of each culture, the presence of cells positive for the T-cell precursor-specific surface markers Joro 37-5 and Joro 75 (11, 16), Thy1 expressed by hemopoietic stem cells and T-lymphocyte lineage cells (17), and the TCR αβ and TCR γδ antigen receptors (18) was assessed by FACS analysis. Also, PCR analysis of DNA from these cells was carried out to determine the presence of rearrangements of the TCR δ, TCR γ, and TCR β genes.

Table 1 (left portion) shows that less than half of freshly isolated mononuclear cells from yolk sac of day 8 and day 8.5 embryos express the PgP-1 adhesion molecule, few express the c-kit receptor, but none express Thy1, Joro 37-5, Joro 75, TCR αβ, or TCR γδ surface markers. PCR analysis of DNA from these cells with appropriate primer combinations did not detect rearrangement of the TCR δ, TCR γ, and TCR β genes (Fig. 2). Upon culture with the EH6 subcapsular thymic epithelial cells, the yolk sac cells, but not cells from the embryo proper, gave rise to Thy1$^+$, c-kit receptor$^+$, Joro

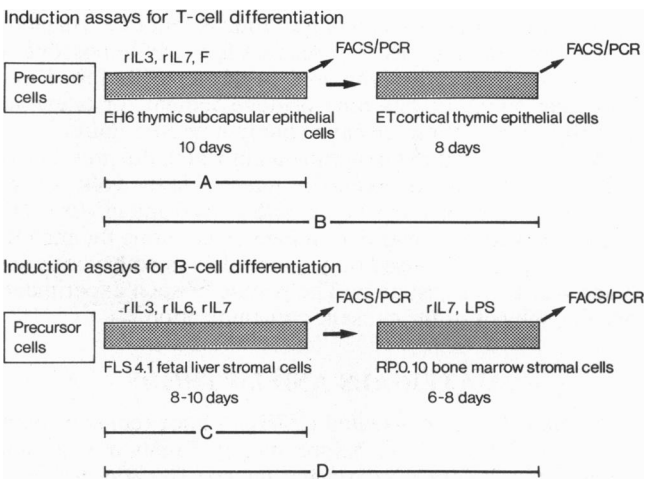

FIG. 1. Schematic illustration of the *in vitro* assays used to induce differentiation of yolk sac mononuclear cells or cells from the embryo proper along the T-lymphocyte (assays A and B) or the B-lymphocyte (assays C and D) pathway.

Table 1. Yolk sac cells differentiate *in vitro* into T- or B-cell lineages

Precursor cell origin	Day	T-cell lineage: Induction assay	% positive cells (FACS analysis) PgP-1	c-kit	Joro 75	Joro 37-5	Thy1	TCR αβ	TCR γδ	B-cell lineage: Induction assay	% positive cells (FACS analysis) PgP-1	B-220	IgM
Yolk sac	8	None	26, 34	8, 12	<1	<1	<1	<1	<1	None	26, 34	<1	<1
Yolk sac	8.5	None	41, 47	10, 14	<1	<1	<1	<1	<1	None	41, 47	<1	<1
Yolk sac	8	A	90, 94	38, 41	18, 27	16, 25	44, 35	<1,<1	4, 3	C	53, 65	15, 21	<1,<1
Embryo	8	A	11, 8	<1,<1	<1,<1	<1,<1	<1,<1	<1,<1	<1,<1	C	13, 7	<1, <1	<1,<1
Yolk sac	8.5	A	91, 96	53, 36	26, 37	27, 33	58, 50	<1,<1	6, 7	C	60, 78	24, 28	<1,<1
Embryo	8.5	A	13, 10	<1,<1	<1,<1	<1,<1	<1,<1	<1,<1	<1,<1	C	12, 11	<1, 2.2	<1,<1
Yolk sac	8	B	95, 98	—	38, 42	38, 41	76, 69	13, 21	25, 16	D	94, 98	38, 43	8, 12
Yolk sac	8.5	B	94, 98	—	35, 40	36, 39	80, 77	16, 19	13, 25	D	99, 96	44, 52	10, 16

The T- and B-lymphocyte precursor potentials *in vitro* of cells from day 8 and day 8.5 yolk sac or embryo proper (whose yolk sac was completely dissected away) of C57BL/6 conceptuses were tested in the *in vitro* systems for T-cell differentiation (Fig. 1, bars A and B) or for B-cell differentiation (Fig. 1, bars C and D). The numbers represent the percentages of positive cells for the surface markers indicated, which were assessed by FACS. Data from two independent experiments are shown. Viable cells recovered (per six-well plate) from yolk sac induced cells at the end of culture were as follows: 1.5–1.8 × 10^6 (culture A), 0.9–1.4 × 10^6 (culture B), 2.1–2.7 × 10^6 (culture C), and 1.1–1.4 × 10^6 (culture D).

37-5$^+$, and Joro 75$^+$ T-cell precursors and some TCR γδ$^+$ T cells, but no cells expressing TCR αβ on the cell membrane (Table 1). PCR analysis of DNA from induced yolk sac cells showed the presence of rearrangements of the TCR δ locus (Vδ1-Jδ1, Vδ4-Jδ1, and Vδ6-Jδ1), cells that have rearranged the Vγ1, Vγ5, Vγ6, or Vγ7 elements of the TCR γ locus, and cells with rearrangements involving the Dβ1 or Dβ2 segments and the Jβ2 gene elements (Fig. 2). In contrast, DNA from cells of the embryo proper cultured under the same conditions did not show such rearrangements in any of the three TCR loci studied (Fig. 2). Taken together, the results indicate that EH6 subcapsular thymic epithelial cells induced yolk sac mononuclear cells to differentiate into pre-T cells and TCR γδ$^+$ cells.

The pre-T cells generated by the yolk sac cells in the first culture (Fig. 1, bar A) were able to further differentiate into TCR αβ$^+$ and to generate more TCR γδ$^+$ cells after culture with monolayers of the cortical thymic epithelial clone ET (Fig. 1, bar B). In contrast, cells from the embryo proper exposed to the same induction assays did not give rise to TCR αβ$^+$ or TCR γδ$^+$ cells (Table 1, left portion). These results show unambiguously that the yolk sac of day 8 and 8.5 C57BL/6 embryos contain precursor cells with potential to develop along the T-lymphocyte pathway *in vitro* after interaction with appropriate thymic epithelial cells.

***In Vitro* Differentiation Along the B-Lymphocyte Pathway.** The potential of yolk sac cells and cells from the embryo proper to differentiate along the B-lymphocyte pathway was also assessed in a two-step culture assay. This induction system utilizes the fetal liver stromal cell line FLS4.1 and exogenous rIL3, rIL6, and rIL7 in a first step (Fig. 1, bar C) and subsequently, the RP010 bone marrow stromal clone, LPS, and rIL7 (Fig. 1, bar D). Both stromal cell lines constitutively produce steel factor (19), the ligand for c-kit receptor (20), which plays a role in the development of lymphocytes in the mouse embryo (19). Differentiation along the B-lymphocyte pathway was determined by FACS analysis using B-220-specific antibody, which detects B-cell precursors and mature B lymphocytes, and IgM-specific antibody, which detects antigen-receptor-bearing B lymphocytes (21), and by DNA-based PCR analysis to assess D–J and V–D–J (variable–diversity–joining) rearrangements of the immunoglobulin heavy chain gene and Vκ–Jκ1 rearrangement of the immunoglobulin κ light chain gene. Table 1 (right

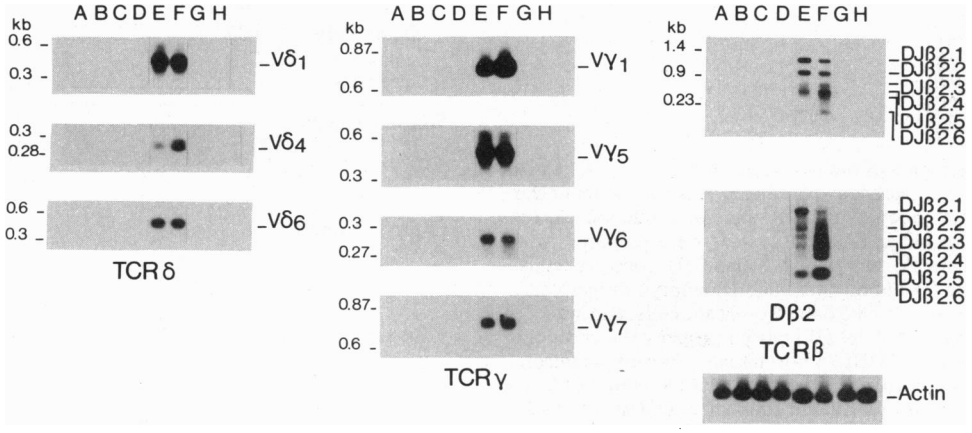

FIG. 2. Rearrangements of TCR δ, γ, and β genes in the progeny of day 8 and 8.5 yolk sac mononuclear cells induced to differentiate along the T-lymphocyte pathway by the induction assay depicted in Fig. 1, bar A. Cell lysates were prepared from uninduced day 8 (lanes A) and day 8.5 (lanes B) yolk sac cells, uninduced day 8 (lanes C) and day 8.5 (lanes D) embryo proper, induced day 8 (lanes E) and day 8.5 (lanes F) yolk sac cells, and induced day 8 (lanes G) and day 8.5 (lanes H) embryo proper cells, and day 15 fetal thymocytes or adult thymocytes (positive controls) and a blank control without DNA template (not shown above for the sake of brevity and simplicity). PCR was used to determine rearrangements of the TCR δ gene (using Vδ1, Vδ4, Vδ6, and Jδ1 primer combinations), the TCR γ gene (using Vγ1, Vγ5, Vγ6, Vγ7, and Jγ1 primer combinations), and the TCR β gene (using 5′Dβ1, 5′Dβ2, and 3′Jβ2 primer combinations) (15). The PCR amplification products were fractionated on agarose gels, blotted to nitrocellulose filters, and hybridized with ^{32}P-labeled Jδ1, Jγ1, or Jβ2 specific probes. The PCR amplification product of the actin gene from the same samples is also shown.

portion) shows that freshly isolated yolk sac mononuclear cells contain no detectable B-220$^+$ or IgM$^+$ B-lineage cells, nor do they have detectable D–J or V–D–J rearrangement of the immunoglobulin heavy chain gene (Fig. 3 and data not shown). Upon culture on FLS4.1 fetal liver stromal cells, rIL3, rIL6, and rIL7, yolk sac mononuclear cells developed into B-220$^+$ cells but not into surface IgM$^+$ mature B lymphocytes. PCR analysis of DNA from induced yolk sac cells documented the presence of cells that have undergone D–J_H1, D–J_H2, D–J_H3, and/or V–D–J_H1, V–D–J_H2, and V–D–J_H3 rearrangements of the immunoglobulin heavy chain (Fig. 3 and data not shown). In contrast, no B-220$^+$ cells or rearrangement of the immunoglobulin heavy chain gene was detected in cells from embryo proper which were cultured under the same conditions (Fig. 3 and data not shown). We have previously found that FLS4.1 stromal cells, although able to support development and growth of fetal liver pre-B cells, are unable to support differentiation of pre-B cells into surface IgM$^+$ B lymphocytes (12). Therefore, the lack of IgM$^+$ B lymphocytes in the yolk sac cells cultured on FLS4.1 stromal cells was not unexpected. However, further culture of the B-cell precursors generated by yolk sac cells in the first step culture (Fig. 1, bar C), on monolayers of the RP010 bone marrow stromal cells, LPS, and rIL7 (Fig. 1, bar D), induced these precursor cells to differentiate into surface IgM$^+$ B lymphocytes (Table 1, right portion) and PCR analysis of DNA from these cells showed the presence of V_κ–J_κ1 rearrangement (Fig. 3). No rearrangement of the κ chain gene was found in uninduced yolk sac cells or in cells from embryo proper either before or after culture on stromal cells, LPS, and rIL7 (Fig. 3). We conclude that yolk sac mononuclear cells from day 8 and day 8.5 C57BL/6 embryos include cells with potential to develop into pre-B cells and IgM$^+$ B lymphocytes after induction with appropriate stromal cells and cytokines *in vitro*.

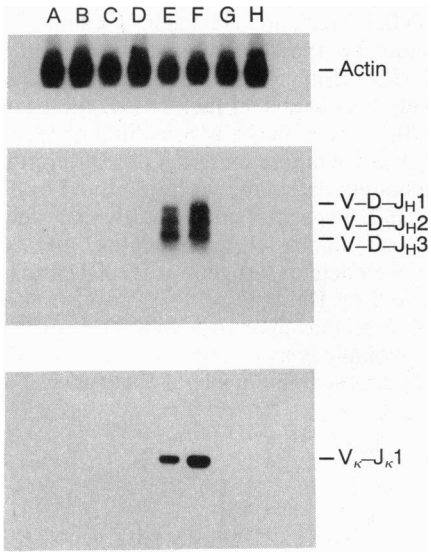

FIG. 3. Rearrangements of the immunoglobulin heavy (H) and κ light (L) chain genes in the progeny of induced yolk sac mononuclear cells along the B-lymphocyte pathway by the induction assays depicted in Fig. 1, bars C and D. Cell lysates were prepared from uninduced day 8 (lane A) and day 8.5 (lane B) yolk sac cells, uninduced day 8 (lane C) and day 8.5 (lane D) embryo proper cells, induced day 8 (lane E) and day 8.5 (lane F) yolk sac cells, and induced day 8 (lane G) and day 8.5 (lane H) embryo proper cells and bone marrow cells from young C57BL/6 normal mice (positive control). The latter and a blank control carried out without template DNA are not shown for the sake of brevity and simplicity. D–J and V–D–J_H and V_κ–J_κ1 rearrangements were analyzed by PCR using D_HL or V_H degenerate and J_H3 or V_κ degenerate and J_κ2 primer combinations as described by Schlissel *et al.* (13, 14). The PCR-amplified products were fractionated on agarose gels, blotted to nitrocellulose filters, and hybridized with ^{32}P-labeled J_H- or J_κ1-specific probes. Sizes of the expected PCR products hybridizing to the J_H probe are ≈1058 (V–D–J_H1), 741 (V–D–J_H2), and 358 (V–D–J_H3) nucleotides and the size of the product hybridizing to the J_κ1-specific probe is ≈536 (V_κ–J_κ1) nucleotides. The PCR amplification product of the actin gene from the same samples is also shown.

Lymphocyte Precursor Potential *in Vivo*. The next set of experiments tested the lymphocyte precursor potential *in vivo* of cells in the yolk sac and embryo proper of day 8.5 C57BL/6 mice. To this end, we obtained cell preparations from whole conceptus (embryo plus yolk sac), embryo proper (embryos in which the yolk sac was completely removed), and yolk sac only. These cell preparations were then directly placed into the spleen, liver, and peritoneal cavity of sublethally irradiated T- and B-lymphocyte-deficient CB17 SCID mice by a surgical procedure previously described (10). Sixteen weeks later, the presence of mature antigen-receptor TCR/CD3$^+$, CD4$^+$, CD8$^+$ T lymphocytes and B-220$^+$ IgM$^+$ B lymphocytes was determined by two-color FACS analysis using H-2^b major histocompatibility complex class I-specific antibody to distinguish lymphocytes that originated from the donor cells (C57BL/6 origin, H-2^b) from cells of host origin (CB17 origin, H-2^d). The results summarized in Table 2 show that only SCID mice that had received cell preparations from whole conceptuses (embryo plus yolk sac) or from isolated yolk sacs had in their spleens donor-derived H-2b$^+$ TCR/CD3$^+$ CD4$^+$, CD8$^+$ T lymphocytes and H-2b$^+$ B-220$^+$, IgM$^+$ B lymphocytes. SCID mice that had received cell preparations from embryos that lacked yolk sac or that received no cell preparations had no detectable H-2b$^+$ donor-derived T or B lymphocytes. Table 2 also shows the percentage of T and B lymphocytes in the spleen of normal young adult C57BL/6 mice used as a positive control for the FACS analysis. We conclude that the yolk sacs of C57BL/6 embryos at day 8.5 of gestation contain precursor cells able to develop *in vivo* into TCR/CD3$^+$ T lymphocytes and IgM$^+$ B lymphocytes. Precursor cells with such potential do not seem to be present in the embryo proper at this stage of mouse development.

Table 2. Lymphocyte precursor potential *in vivo*

Donor cells day 8.5 C57BL/6	CB17 SCID host	% lymphocytes in spleen (FACS analysis)			
		T lymphocytes		B lymphocytes	
		H-2b$^+$/ TCR CD3$^+$	H-2b$^+$/ CD4$^+$ CD8$^+$	H-2b$^+$/ B-220$^+$	H-2b$^+$/ IgM$^+$
Whole conceptus	1	21.4	17.6	18.0	12.2
	2	25.1	23.8	22.7	16.1
	3	7.5	5.3	20.5	13.7
	4	19.1	18.5	34.3	30.6
Embryo proper	1	<1	<1	<1	<1
	2	<1	<1	<1	<1
	3	<1	<1	<1	<1
	4	<1	<1	<1	<1
Yolk sac	1	12.8	10.5	11.6	9.3
	2	24.6	20.3	21.4	17.5
	3	9.8	8.2	17.7	15.2
	4	11.2	7.9	25.9	22.5
None	n = 3	<1	<1	<1	<1
Normal spleen	n = 3	37.0	36.2	57.8	57.2

In vivo lymphoid precursor potential of whole conceptuses (embryo + yolk sac), embryo proper (lacking yolk sac), and yolk sac from day 8.5 (7–12 somite pairs) C57BL/6 mouse embryos was studied by directly transferring the cells into sublethally irradiated T- and B-lymphocyte-deficient SCID mice. Twelve to sixteen weeks later the presence of T- and B-lymphocyte-lineage cells in these mice was determined by two-color FACS analysis.

Our studies *in vivo* and *in vitro* with cell preparations from day 8 and day 8.5 embryos provide evidence that the yolk sac contains precursor cells with lymphocyte potential at a time of development when such cells could not be detected in the embryo proper. Also, our results, obtained with embryos whose age was precisely determined and before blood circulation had started, rule out the possibility that the lymphoid precursor cells detected in the yolk sac were circulating cells that originated elsewhere, which could not be excluded in previous *in vivo* studies carried out with day 9 or older mouse embryos (22–25).

The cells in the yolk sac at day 8–8.5 of mouse development that show T- and B-lymphocyte precursor potential are most probably multipotent progenitors or even primitive hemopoietic stem cells rather than pro-T- or pro-B-lymphocyte lineage-determined progenitors. In support of this view are the findings that yolk sac mononuclear cells at this time of gestation express PgP-1 and c-kit but are negative for surface markers of T- or B-lymphocyte progenitors (i.e., Joro 37-5, Joro 75, B-220) and have their TCR γ, δ, and β genes and immunoglobulin genes in the germ-line configuration. The yolk sac stem cells most likely become cell-determined lymphocyte progenitors within the embryo (e.g., in the liver) at later stages upon the proper cell–cell interactions.

The results described here are at odds with the claim that B-cell precursors appear first in the embryo proper (8). As that study was carried out with "day 9.5–10" embryos, we suspect that the B-cell precursor activity detected in cell preparations from the embryo reflects the presence of circulating hemopoietic cells that have started B lymphopoiesis in the developing liver. The stromal cell line and the culture conditions used by these workers might not be able to induce B-cell differentiation from very early hemopoietic precursors, such as those found in yolk sac.

As proposed by Moore and Owen (26) 25 years ago, lymphohematopoietic precursor cells present in the yolk sac will enter the bloodstream at day 9 of mouse development and consequently reach a widespread tissue distribution. As organs with specialized microenvironments that support growth and differentiation of such precursor cells along the lymphocyte pathways develop fully (e.g., liver and thymus), these cells and their immediate progeny will expand and differentiate in such highly supportive microenvironments. Precursor cells that ended up in tissues that are not supportive are destined to die. The findings with day 8 and day 8.5 mouse embryos described here are consistent with this model.

It will be important now to determine the site of origin of the cells that eventually give rise to yolk sac lymphohemopoietic precursors. In chicks, the first B-lymphocyte-committed lineage cells are found in the yolk sac at day 5–6 of development (27), although the earliest identifiable cells with hemopoietic precursor potential are found within the aortic mesoderm at day 3–4 of gestation (28). We hope that the *in vitro* systems that support T- or B-lymphocyte development from yolk sac cells described here will also be useful to determine the site, origin, and nature of the cells that give rise to yolk sac lymphohemopoietic precursor cells in the mouse embryo. These *in vitro* systems should also facilitate the study of the molecular events leading to lymphocyte lineage commitment in the mouse embryo.

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