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Emergence of multipotent hemopoietic cells in the yolk sac and paraaortic splanchnopleura in mouse embryos, beginning at 8.5 days postcoitus

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ABSTRACT **We show by an *in vitro* approach that multipotent hemopoietic cells can be detected in the body of the mouse embryo between the stages of 10–25 somites (8.5–9.5 days of gestation)—i.e., prior to liver colonization (28–32 pairs of somites). Interestingly, hemopoietic cells appear in parallel in this location, the paraaortic splanchnopleura, and in the yolk sac, where they represent a new generation by reference to the primitive hemopoietic stem cells. Lymphoid cell clones, which could differentiate into mature B cells, were obtained from yolk sac and paraaortic splanchnopleura cell preparations but not from other tissues of the embryonic body. These B-cell precursors were first detected around the stage of 10 somites; thereafter, their initial minute numbers increased in parallel in the yolk sac and the paraaortic splanchnopleura, suggesting that their emergence in the two sites was simultaneous. By single cell manipulation, we show that these precursors can generate B and T lymphocytes and myeloid cells; these precursors can thus be defined as multipotent hemopoietic cells.**

Hemopoietic precursors seed the mouse fetal liver between the stages of 28–32 pairs of somites (S) at 10 days postcoitus (dpc) (1–3). The embryonic thymus is colonized somewhat later, starting at 10.5 dpc (4–6). The origin of these precursors is controversial. In birds, definitive hemopoietic stem cells originate from the region neighboring the dorsal aorta (7), as shown in chimeras between a quail embryo and a chicken yolk sac (YS). The cells colonizing the hemopoietic rudiments and giving rise to the definitive erythroid lineage were found to be exclusively of intraembryonic origin (reviewed in ref. 8).

In the mouse, hemopoietic cells, capable of reconstituting lethally irradiated recipient mice, have been detected in the YS of 8- to 10-dpc embryos (9–12) and in the caudal region of the embryo proper at 9 dpc (12). Cells capable of undergoing *in vitro* differentiation into B lymphocytes have been detected in the YS (13–15) and in the embryonic body at 9 dpc (13, 14, 16). The origin of these precursors in the body could be restricted to the paraaortic splanchnopleura (P-Sp) (17). Transplants of this structure were shown capable of generating mature B cells in Scid mice, when taken from 10- to 25-S embryos. Long-term reconstitution of iradiated mice was obtained from precursors present in the AGM (aorta, gonad, mesonephros) region (18)—i.e. slightly older tissues (31–48 S) derived from the P-Sp.

We now report *in vitro* experiments that probe the differentiation potential of the first precursors appearing in the P-Sp. We used a culture system that allows the proliferation of uncommitted precursors and their differentiation into the myeloid and B-cell pathways (19).

We isolated cells from the YS, P-Sp, and remainder of the embryonic body from embryos between 8.5 dpc (10 S) and early 10 dpc (25–30 S). B-lymphocyte precursors were first detected both in the P-Sp and in the YS at the stage of 10 S and their numbers increased in parallel in both locations. By the stage of 25 S, we could detect 15 precursors in each location. The remainder of the embryonic body was consistently negative.

Using stringent limiting-dilution conditions or single cell micromanipulation, we show further that precursors detected at these stages are multipotent hemopoietic cells.

MATERIALS AND METHODS

Cell Preparations. C57BL/6 (Ly5.2), BALB/c (Ly5.2), and C57BL/6 (Ly5.1) mice were kept in the colony of the Pasteur Institute. Mature females were caged with breeding males; the day of plug observation was considered day 0 of gestation. Ly5.2 embryos (BALB/c or C57BL/6 strains) were used to dissect the P-SP, the YS, and the remainder of the embryonic body. Single cell suspensions were prepared by passage of the selected tissues through a 26-gauge needle. Viable cells were counted by trypan blue exclusion.

Culture Conditions. Culture conditions have been described (19, 20). S17 stromal cells [from K. Dorshkind, University of California, Riverside (21)] were plated in 96-well plates (500–1000 cells per well), cultured overnight at 37°C in 5% CO_2/95% air, and irradiated (2000 Gy) with a cesium source.

Cells were seeded at limiting dilution, using 48–96 wells from 96-well plates for each cell concentration. The culture medium was supplemented with the following cytokines: interleukin 7 (IL-7) (50–100 units/ml) and IL-3 (1:200 dilution) both obtained from the supernatants of stably transfected cell lines (from F. Melchers, Basel); kit ligand (KL) (Genetics Institute, Boston) was used at a 1:500 dilution, a dilution that allows the emergence of mast cells from adult bone marrow (data not shown).

Half of the medium was replaced every 5 days by fresh medium containing IL-7, IL-3, and KL. At day 10 or 14 of culture, clones were harvested from the wells containing lymphoid cells and each was divided in two or three lots. One was cultured on stromal cells in the presence of IL-3, KL, and granulocyte–macrophage colony-stimulating factor (GM-CSF) at a 1:100 dilution (obtained from C. Roth, Pasteur Institute) (myeloid culture conditions). Another lot was cultured on S17 stromal cells with IL-7 (B-cell culture conditions). In cases in which a third lot was prepared, it was placed in fetal thymus organ cultures (FTOC, see below).

For analysis of mature B cells, cells grown in the B-cell culture conditions were replated for 2 or 3 days on a fresh S17 feeder layer without IL-7. Mitogen stimulation was performed

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Abbreviations: FTOC, fetal thymus organ culture; P-Sp, paraaortic splanchnopleura; S, pairs of somites; YS, yolk sac; dpc, days postcoitus; IL, interleukin; GM-CSF, granulocyte–macrophage colony-stimulating factor; LPS, lipopolysaccharide; FACS, fluorescence-activated cell sorter; TcR, T-cell receptor.

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by adding lipopolysaccharide (LPS) (25 μg/ml) to 5000 cells plated on irradiated S17 cells in 96-well plates (*Salmonella typhosa* W0901; Difco).

ELISAs. Immunoglobulin production was tested by ELISA. Nunc-Immuno plates MaxiSorp (GIBCO) were coated with purified antibody from allotype-specific hybridomas AF6 (anti-mouse Igh6b) or RS3.1 (anti-mouse Igh6a). After blocking with phosphate-buffered saline (PBS) and 2% bovine serum albumin, the culture supernatant was serially diluted. Plates were incubated first with the appropriate dilution (19) of anti-mouse μ chain coupled to horseradish peroxidase (Sigma) and then with 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) as substrate. The absorption was read at 405/603 nm.

To ascertain that the detected precursors were not of maternal origin, we used C57BL/6 (IgM^b) × BALB/c (IgM^a) F_1 embryos, and the immunoglobulin allotype was routinely identified in the supernatant of the LPS-stimulated cells.

FTOC. Thymic lobes were dissected from 15-dpc C57BL/6 (Ly5.1) mice. FTOCs were made as described (22), except that the depletion of endogenous lymphoid cells was performed by irradiation (2500 Gy) instead of deoxyguanidine treatment. Briefly, 30 μl of cell suspension was placed together with an irradiated fetal thymic lobe in each well of a Terasaki plate and cultivated in a hanging drop for 24–48 hr. Cells from each clone (see above) were split between 6 FTOCs. Colonized thymic lobes were cultured for a further 10–13 days on polycarbonate filters (0.8 μm; Merck) floating on hemostatic sponges (Helistat, Collatec, Plainsboro, NJ) embedded in culture medium (Opti-MEM; GIBCO) supplemented with 10% fetal calf serum. To analyze cells from repopulated thymuses, single cell suspensions were made by teasing the organs with two needles. Cells obtained from several thymic lobes repopulated with cells from the same clone were pooled for fluorescence-activated cell sorter (FACS) analysis.

Cytofluorometric (FACS) Analysis. Cell suspensions were stained for 20–30 min with the following antibodies: for B-cell characterization, $F(ab')_2$ goat anti-mouse μ chain coupled to fluorescein (Jackson ImmunoResearch) and either anti-B220 (purified supernatant from 14.8 hybridoma line) or anti-Ly1 (Becton Dickinson) coupled to biotin followed by streptavidin coupled to phycoerythrin; for T-cell staining, anti-Ly5.2 antibody purified from the supernatant of the hybridoma line 104.2 coupled to fluorescein and anti-CD4 (Becton Dickinson) or anti-TcRαβ (Pharmingen), both coupled to phycoerythrin. All analyses were performed by double staining, and propidium iodide was used to mark dead cells. FACS analysis was done in a FACScan with the LYSIS II program (Becton Dickinson).

RESULTS

Location and Quantitative Evolution of B-Cell Precursors Between 8.5 and 9.5 dpc (10–25 S). Embryos were precisely staged and grouped within a 5-S range. Each experimental group comprised 20–30 embryos. After separation of the YS, the P-Sp was dissected from the remainder of the body. The P-Sp comprises developing gut (including the septum transversum in which the fetal liver develops), the endothelium of blood vessels (the paired dorsal aortae and the omphalomesenteric artery), and the splanchnic mesoderm (Fig. 1). Single

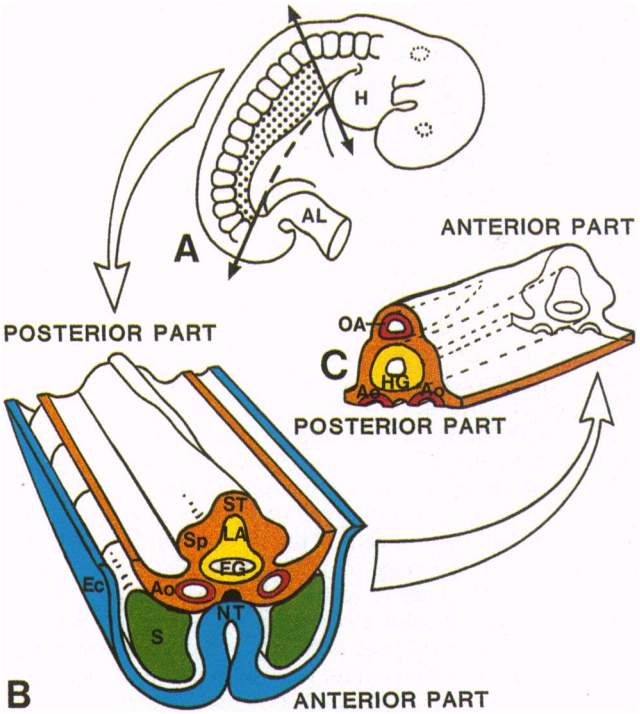


FIG. 1. Schematic representation of dissection of the P-Sp (stippled) from a 9-dpc mouse embryo (15–20 S). (*A*) Overall morphology of a 9-dpc embryo after removal of the YS. Anterior and caudal parts are cut off from the embryo along the arrows. AL, allantois; H, heart. (*B*) Schematic anatomy of the truncal part of the embryonic body (ventral view). Ao, aorta; Ec, ectoderm; FG, foregut; LA, liver anlage; NT, neural tube; S, somite; SP, splanchnopleural mesoderm; ST, septum transversum. (*C*) Schematic representation of explanted P-Sp. HG, hindgut; OA, omphalomesenteric artery.

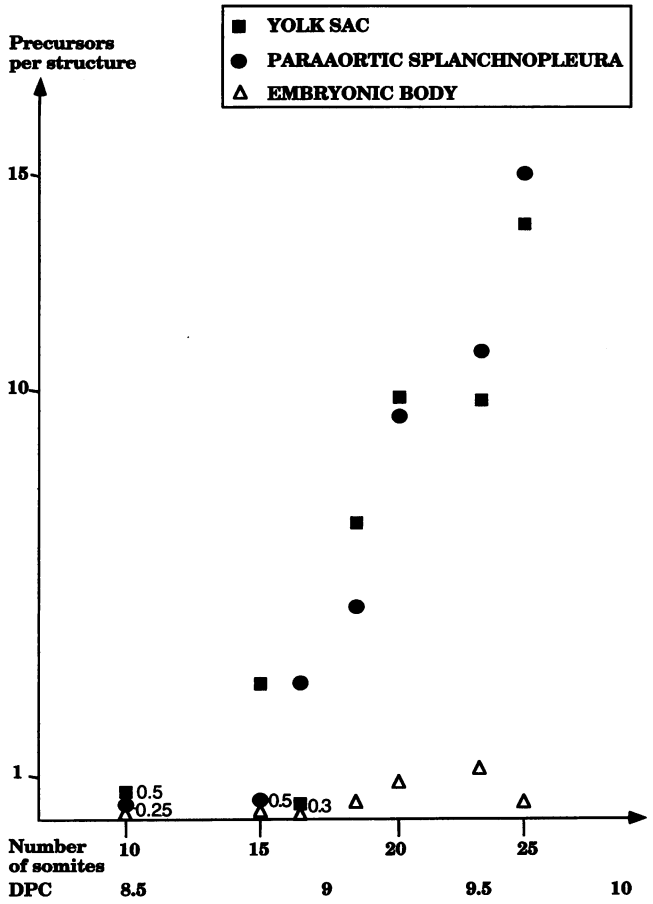


FIG. 2. Quantification of B-cell precursors found in the YS, the P-Sp, and the remainder of the embryonic body at different developmental times. Whenever precursor number was <1 per region, the actual value appears next to the symbol. When representing the progenitor number, the mean value of somite numbers in the group of embryos analyzed was chosen—e.g., the two experiments shown at 15 S both involved embryos belonging to a 13- to 18-S range. Each cell suspension was prepared from 20–30 pooled embryos.

Table 1. Frequency of lymphoid precursors in YS and P-Sp of mouse embryo at different developmental stages

Stage	Structure	No. of cells per structure	Frequency of lymphocyte clones
10–18 S	YS	6,400	1:2,000
	P-Sp	1,100	1:200
	Embryo body*	12,000	<1:12,000
16–20 S	YS	11,466	1:1,400
	P-Sp	1,500	1:120
	Embryo body*	20,000	1:20,000
18–26 S	YS	13,320	1:900
	P-Sp	1,000	1:100
	Embryo body*	25,000	<1:25,000

*Embryo deprived of both YS and P-Sp.

cell suspensions were prepared from each of these two embryonic fractions and from the YS. Viable cells were plated under limiting dilution conditions on S17 stromal cells in the presence of IL-7 at 10, 30, and 100 cells per well for the P-Sp and YS and at 100, 300, and 1000 cells per well for the embryonic body. The presence of pre-B-cell clones was detected by microscopic inspection after 15–20 days in culture and was confirmed 10 days later by identification of immunoglobulin secreted after LPS stimulation.

Fig. 2 shows the numbers of B-cell precursors detected in the YS, P-Sp, and remaining embryonic bodies, between stages 10 and 30 S. B-cell precursors were never detected before the 10-S stage (8.5 dpc) (three or four experiments). Very low numbers of B-cell precursors, usually B-cell precursors steadily increased in the YS and in the P-Sp. Nowhere else in the body of the embryo could B-cell precursors be detected.

There is a 100- to 500-fold difference between the splanchnopleural region and the YS in the frequency of B-cell precursors (Table 1). At 18–26 S, the frequency of lymphocyte precursors in the P-Sp can reach 1 per 100 viable cells. Precursors for further characterization were obtained from these stages.

Pluripotency of Precursors Obtained from 20- to 25-S Embryos. In 12-dpc fetal liver, half of the B-cell precursors are bipotent uncommitted cells that can generate macrophages and B cells (20). We were therefore interested in determining the differentiation capacity of the precursors detected at 8.5–9.5 dpc. Cells isolated from the P-Sp and from the YS of embryos around the 20- to 25-S stage (three independent experiments) were plated at 10 cells per well; 2.2% of the wells were positive for growing clones—namely, of 768 wells seeded, 17 contained one clone in which lymphoid cells were detected. After 10–15 days in culture, the clones were split in two. Half of each clone was tested for myeloid cell differentiation. After a further 15 days in culture with GM-CSF, KL, and IL-3, macrophages, megakaryocytes, and granular polymorphonuclear cells were present. The other half was replated on S17 cells in the presence of IL-7 to support pre-B-cell growth and differentiation. Cells grown in these conditions were stimulated with LPS. An ELISA allowed detection of immunoglobulin. The embryonic origin of all B-cell-containing clones was ascertained by the detection of both IgM^a and IgM^b allotypes from F_1 embryos.

The 17 analyzed clones contained B-lineage cells and multiple myeloid cell types; such precursors were isolated from both YS and P-Sp.

To rule out cell–cell adhesion and because the low frequency of the event measured did not allow unambiguous calculations, we micromanipulated 100 single cells into individual wells of a 96-well plate. Cells isolated from the P-Sp at 25 S were enriched by panning for the expression of the AA4.1 surface marker. This marker was previously shown to be expressed in all B-cell precursors isolated from total embryos at this stage of gestation (19). Of the 100 micromanipulated cells, six clones containing lymphocytes were obtained and split into myeloid and lymphoid culture conditions, as described above (Fig. 3). Clones containing cells with lymphocyte morphology were expanded and subsequently double-stained for surface markers IgM/B220 and IgM/CD5. The FACS profiles of one of these clones (Fig. 4*A*) show

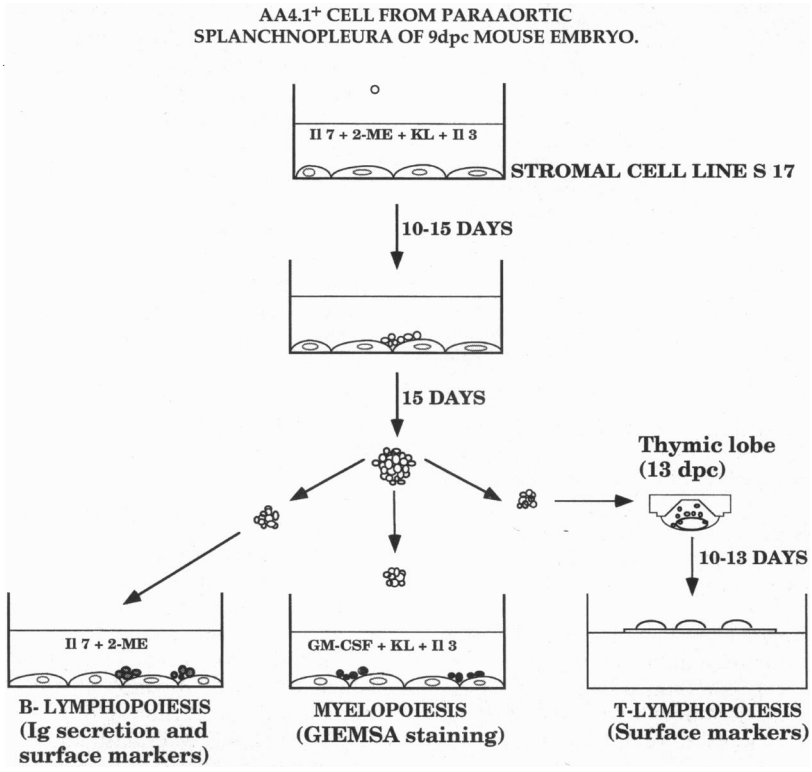
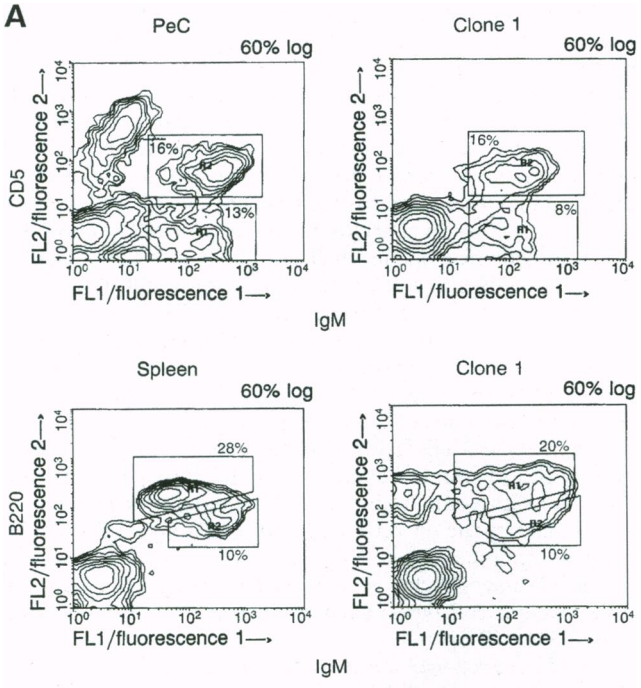


FIG. 3. Schematic illustration of *in vitro* culture conditions used to detect myeloid and B- and T-lymphoid potentialities of single micromanipulated cells. Note that the experimental series involving FTOC cultures were initiated with 10 unsorted cells. 2-ME, 2-mercaptoethanol.



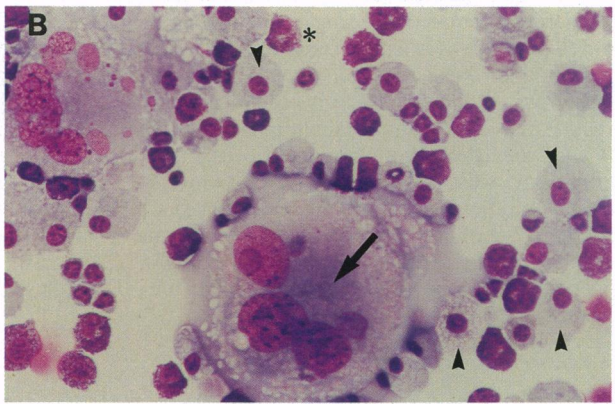


FIG. 4. (*A*) FACS profile of expanded B-cell precursors corresponding to one of the analyzed clones (clone 1). Staining profiles of cells from clone 1 were compared to staining profiles of cells from the peritoneal cavity (PeC) and spleen cells of adult mice as a source of B1a, B1b, and B2 B cells. Clone 1 is representative of clones obtained by micromanipulation in that all clones gave profiles similar to the ones shown here at different periods of culture. (*B*) May–Grünwald–Giemsa staining of cells from clone 1 growing in myeloid conditions. Arrow, megakaryocyte; arrowheads, macrophages; asterisk, neutrophil. After 20 days in culture, cells were divided into myeloid and lymphoid conditions and after a further 10–20 days in culture, cells were spotted on a slide and stained.

that cells bearing IgM on their surface could be detected. This is also true for the other five clones so that one was chosen as a representative example. The surface immunoglobulin-positive cells were heterogeneous as to the expression of CD5/Ly1 and expressed different levels of B220. Those surface phenotypes are characteristic of B1a and B1b B-cell subsets. Moreover, the presence of $B220^{high}/IgM^{low}$ cells strongly suggest that B2 cells are also present.

All six clones were capable of immunoglobulin secretion upon LPS stimulation. Titration of immunoglobulin present in the supernatant shows that secretion ranged from 2 to 100 μg of IgM per ml (i.e., 100-fold less immunoglobulin than normal mouse serum). Unstimulated cells were unable to secrete immunoglobulin.

Cells from the same clones growing in myeloid conditions contained myeloblasts, megakaryocytes, macrophages, basophils, and erythroblasts (Fig. 4*B*). We conclude that precursors present in the P-Sp are multipotent hemopoietic cells.

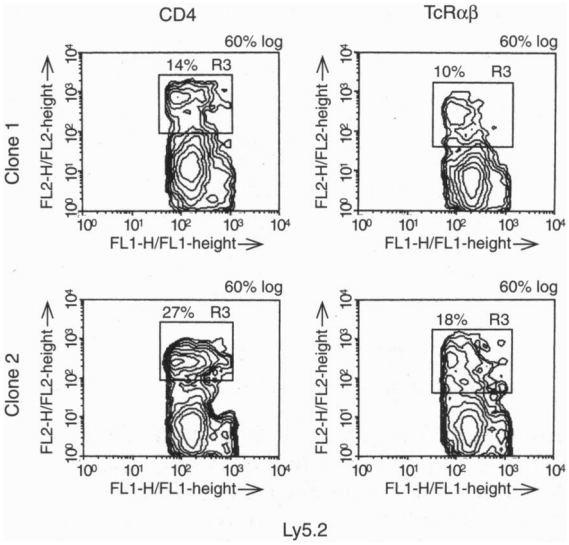


FIG. 5. FACS profile of cell suspensions prepared from five pooled thymic lobes repopulated with cells from either clone 1 or clone 2 previously analyzed for myeloid and B-lymphoid potentialities. Profiles are shown after gating donor-derived cells that can be identified by staining with anti-Ly5.2 antibody.

Cells from the P-Sp Can Generate T Cells in FTOCs. The limiting dilution method followed by FTOC was used to test the T-lymphoid potential of the pluripotent precursors obtained on the S17 stromal cell line.

Cells isolated from the P-Sp of C57BL/6 embryos (Ly5.2) at stage 20–25 S were plated (10 cells per well) on S17 stromal cells with KL and IL-7. Ten clones were obtained, of which two clones that contained B lymphocytes were chosen. At day 14 of culture, cells were divided into myeloid and B-cell growth conditions. A third lot was added to embryonic thymic lobes from 15-dpc C57BL/6 (Ly5.1) embryos (1–2 $\times$ 10^4 cells per lobe) in a hanging drop culture. After 12 days of culture, cells from repopulated thymuses were analyzed for surface expression of the donor Ly5.2 marker and either CD4 or T-cell receptor (TcR)$\alpha\beta$ in double staining.

Fig. 5 shows the cytofluorometric profile of cells repopulating five lobes. We detected 13% and 28% of $CD4^+$ cells in lobes cultured, respectively, with cells from clones 1 and 2, and 7% and 15% of TcR$\alpha\beta^+$ cells, showing the potential of those precursors to generate T lymphocytes. Cells from the same clones became unable to generate T cells after an additional 3 weeks in culture (data not shown).

These results demonstrate that multipotent hemopoietic precursors isolated from the P-Sp at stage 20–25 S have the potential to generate T cells after 2 weeks of expansion with IL-7 and KL on the S17 stromal cells. This potential is lost after longer periods of culture *in vitro*.

DISCUSSION

Using an array of culture conditions, we demonstrate the simultaneous emergence of hemopoietic precursors in the YS and in one particular site of the mouse embryonic body—namely, the P-Sp. The presence of hemopoietic precursors in this location has so far been shown by an *in vivo* assay (17), which uncovered B-lymphoid potentialities in the P-Sp but did not allow the quantitation of precursor numbers. The present report highlights several findings concerning the emergence of precursors in the 10- to 25-S mouse embryo.

(*i*) The P-Sp is the only region in the embryonic body producing B-cell precursors; (*ii*) the kinetics of emergence of

these cells from the P-Sp is established; (*iii*) the YS produces progenitors with B-cell potential according to a parallel time course; (*iv*) the progenitors that appear in the P-Sp are multipotent.

There has been a controversy in the literature around the first site harboring lymphoid precursors, during embryogenesis. According to Ogawa *et al.* (16), cells capable of undergoing immunoglobulin gene rearrangements appear in the embryonic body at 9.5 dpc and in the YS at 10 dpc only. According to Palacios and Imhof (15), B-cell precursors are present only in the YS at 9 dpc. By means of the culture conditions used here, we previously detected such precursors in both the YS and the whole embryonic body (with the P-Sp) at about the same stages (13). Huang *et al.* (14) confirmed this result by using a similar culture system.

We presently circumscribe the region containing these precursors to the P-Sp and show that, around the 10-S stage, <1 precursor can be detected per embryo. Individual experiments allow the detection of B-cell precursors in the YS or the paraaortic region of the embryo proper. Thus, small variations in the culture conditions could result in the absence or presence of precursors, reflecting a stochastic variability as different progenitors emerge.

Because the blood circulation between the YS and the embryo body becomes established at the 8-S stage (F.D.-L., unpublished observation), we cannot warrant the origin of the detected precursors. We suggest that at the 10- to 15-S stage hemopoietic precursors endowed with multipotentiality are starting to appear in both YS and P-Sp simultaneously. Their uneven distribution indicates that they do not become dispersed through the blood flow after emerging in a single of these two sites.

It has recently been shown that at 10 dpc (35 somites) the region of the embryo that comprises the aorta, the genital ridges, and mesonephros (AGM) harbors cells capable of long-term, multilineage reconstitution potential (18). Although this work did not detect such a potential before 10 dpc, we think that the stem cells revealed in the AGM are related to the multipotent precursors present in the P-Sp, since the AGM derives from this region.

The stage of gestation when lymphocyte precursors are first detected coincides with the earliest stage when *in vivo* reconstitutions of the hemopoietic system could first be achieved by using cells from mouse YS (9–12) or embryonic bodies (12, 18). This result argues that cells that can reconstitute irradiated recipients and cells capable of differentiating into lymphocytes *in vitro* appear at the same time during embryogenesis.

We recently demonstrated that cells from the P-Sp, when grafted under the kidney capsule of Scid mice, generated B cells of the B1a phenotype (17). It is likely that the microenvironment of the P-Sp maintained in grafting experiments exerts a restriction on the fate of the B-cell progenitors, since the present *in vitro* findings show that at least two B-cell subsets (B1a + B1b), and also cells that share surface markers with the B2 subset ($B220^{high}IgM^{low}$), can be generated from a unique precursor present at this early stage of embryonic development.

Even more revealing is the potential to generate T lymphocytes in FTOC, since we never detected cells with surface expression of either CD4/CD8 or TcR$\alpha\beta/\gamma\delta$ (I.G. *et al.*, unpublished observations) in the clones grown from paraaortic cells on the S17 feeder layer. Similarly, Liu and Auerbach (23) state that cells from the 8- to 9-dpc YS that can repopulate fetal thymus do not express T-lineage markers, whether freshly isolated or cultivated *in vitro*. We can, therefore, consider that some cells maintain hemopoietic multipotentiality in our cultures for as long as 2 weeks. Indeed the S17 stromal cell line supports the *in vitro* self-renewal of primitive stem cells capable of long-term reconstitution (24).

We speculate that, while the multipotent hemopoietic cells that arise early in the YS (at 7.5 dpc) engage in erythropoiesis, the second wave of hemopoietic cells that appear around 8.5–9 dpc simultaneously in the P-Sp and the YS are responsible for definitive hemopoiesis. The fetal liver and the thymus are probably seeded by migrants from the P-Sp. Multipotent cells in the YS could either circulate to the hemopoietic organs and intermix with the P-Sp cells or never reach the hemopoietic organ rudiments, as experimental evidence demonstrates in birds (8). The respective contributions to liver and thymus colonization of the precursors from these two locations in mouse embryo remain to be determined, a problem for which experimental strategies must be devised.

The assay system used here should allow us to clarify further the question of lineage relationship and differentiation potential of hemopoietic stem cells that become committed during early development.

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