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Intraembryonic, but Not Yolk Sac Hematopoietic Precursors, Isolated before Circulation, Provide Long-Term Multilineage Reconstitution

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Summary

The relative contribution of yolk sac and intraembryonic precursors to hematopoiesis has been a matter of long-standing controversy. As reconstitution activity has so far only been found in embryonic tissues after the onset of circulation, the origin of reconstituting cells could not be formally established. Here, we separated yolk sac and intraembryonic splanchnopleura prior to circulation and maintained the explants in organ culture before transfer. Precursors derived from the intraembryonic site generated multilineage hematopoietic progeny in adult mice for more than 6 months. Yolk sac cells only provided myeloid short-term reconstitution. The results reveal a differential hematopoietic capacity of precirculation embryonic tissues in vivo, and indicate that the only cells capable of adult long-term hematopoiesis are of intraembryonic origin.

Introduction

During vertebrate embryogenesis, the first hematopoietic cells appear in the yolk sac (YS) blood islands. The major hematopoietic organs (fetal liver and bone marrow in mammals) require the input of exogenous hematopoietic precursors to generate a differentiated progeny. It was thus suggested that hematopoietic stem cells (HSC) originate in the extraembryonic YS and later migrate to the fetal liver (Metcalf and Moore, 1971). This conclusion prevailed until studies using chick-quail (Dieterlen-Lièvre, 1975) and amphibian (Turpen et al., 1981) chimera systems showed the existence of an intraembryonic site harboring hematopoietic cells.

In mice (Godin et al., 1995, 1999) and humans (Tavian et al., 1999), the equivalent intraembryonic region, which comprises the dorsal aorta and surrounding splanchnic mesoderm, the paraaortic splanchnopleura (P-Sp; 8.5–10 dpc; days postcoitum), also displays hematopoietic activity. At later stages of development (10–12 dpc), the aorta, gonads, and mesonephros have developed within the P-Sp, and this structure is called the AGM (Medvinsky and Dzierzak, 1996; Müller et al., 1994).

An outstanding question concerns the independent origin of multipotent hematopoietic stem cells. YS hematopoiesis is mainly supporting primitive erythrocyte production. These erythroid cells are nucleated and express embryonic globins. Multipotent progenitors are also present in the YS after 8.5 dpc (Godin et al., 1995; Huang et al., 1994; Nishikawa et al., 1998; Weissman et al., 1978). At this stage, P-Sp/AGM also contains multipotent hematopoietic precursors (Godin et al., 1995), reaching a maximal number at 10.5–11.5 dpc (Godin et al., 1999), concomitant with the onset of fetal liver colonization by HSCs (Houssaint, 1981). From 10.5 dpc, long-term-reconstituting (LTR) hematopoietic precursors appear in the P-Sp/AGM, while YS cells only show comparable activity after 11 dpc (Huang and Auerbach, 1993; Medvinsky and Dzierzak, 1996; Müller et al., 1994).

Direct intrahepatic transfers of $CD34^+$ precursors isolated from 9 dpc embryos in newborn recipients showed the existence of HSCs in both the P-Sp and YS (Yoder et al., 1997). As a higher number of cells endowed with LTR activity can be collected from the YS than from the P-Sp, it was argued that a de novo generation of HSC may occur in the 9 dpc YS (Dzierzak et al., 1998). All of these experiments, performed when hematopoietic precursors can freely circulate between the extra- and intraembryonic compartments (8 dpc, 5 S; somite), did not show independent generation of HSCs.

Avian (Dieterlen-Lièvre, 1975) and amphibian (Turpen et al., 1981) chimera systems, in which the intra- and extraembryonic compartments were reassociated before the establishment of circulation, showed an independent origin of hematopoietic populations in both sites. Furthermore, in amphibian embryos, this independent origin could be traced back to the early blastomere stages (Ciau-Uitz et al., 2000). In both chimeric systems, it was shown that circulating YS-derived hematopoietic cells were rapidly replaced by those of intraembryonic origin. No lymphocytes were detected in the progeny of YS cells, except for a small contribution to thymocytes in amphibian (Bechtold et al., 1992).

We previously isolated the YS and intraembryonic splanchnopleura (Sp) from mouse embryos before blood circulated, and compared the in vitro differentiation potential of hematopoietic precursors generated at both sites. This experiment revealed striking differences between the precursors from the two compartments. Unlike the Sp, the YS cells were unable to generate a lymphoid progeny. They also displayed a reduced potential for maintenance and differentiation of myeloid lineage progenitors. Moreover, we performed mixed cultures of YS cells from Ly5.2 C57BL/6 embryos with Sp cells from embryos of the congenic Ly5.1 strain. All lymphocytes generated in such cultures expressed the Ly5.1 marker, showing that they were of Sp origin. These experiments suggested that the Sp and YS environments could not reciprocally influence the differentiation potential of these tissues in vitro (Cumano et al., 1996).

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In these experiments, we could not evaluate the contribution of YS and Sp cells to definitive hematopoiesis due to the inability of these early hematopoietic precursors to achieve in vivo reconstitution (Cumano et al., 1996; Godin et al., 1995). The failure of Sp and YS cells to repopulate an adult hematopoietic compartment could result from: (1) an intrinsic inability to achieve LTR; (2) a disadvantage during a competitive repopulation assay in the presence of adult hematopoietic progenitors; (3) a limiting number of available HSC; or (4) the low expression of MHC class I in embryos before 10.5 dpc (Jaffe et al., 1991; Ozato et al., 1985), making these early hematopoietic precursors potential targets of host-derived natural killer (NK) cells.

Here, we attempted to identify the origin of HSCs using a cell transfer scheme in which recipient mice were either deprived of T and B cells (*Rag2*$^{-/-}$) or were also deficient in NK cells (*Rag2*$\gamma c^{-/-}$). In order to increase the number of precursors generated in the YS and Sp explanted during precirculatory stages, we implemented a 4 day organ culture step. This approach could also provide information on a possible second generation of hematopoietic precursors by the YS. We found that 6 to 8 months posttransplantation, donor-derived multilineage reconstitution was observed in alymphoid *Rag2*$\gamma c^{-/-}$ mice injected with Sp-derived cells. Recipients of YS cells showed only short-term erythro-myeloid reconstitution. This result establishes in mammals that early intraembryonic precursors can engraft an adult environment and that they are the only cells at this stage capable of long-term, multilineage reconstitution of the hematopoietic system.

Results

Splanchnopleura Autonomously Generates Hematopoietic Precursors in Organ Culture

We have previously reported that Sp, freshly isolated before the 8.5 dpc (12 S) stage, does not contain a detectable frequency of hematopoietic precursors (Godin et al., 1995). However, after 2 days in organ culture, both erythro-myeloid and lymphoid progenies were obtained in about 70% of Sp explants if the respective cell suspensions further differentiated with stromal cells and exogenous cytokines (Cumano et al., 1996).

Here, we initially defined the organ culture conditions that elicit the detection of hematopoietic progenitors in a maximal number of Sp and YS explants. Sp and the corresponding YS were isolated from embryos in which blood circulation between the extra- and intraembryonic compartments is not yet established (7.5–8 dpc, 0–5 S; A. Manaia and I.G., unpublished data). Both hemogenic sites were also dissected during the 5–10 S stage, which corresponds to the period extending from the beginning of the circulation of YS-derived erythrocytes and the onset of intraembryonic precursor production (8.5 dpc, 12–15 S; Godin et al., 1995). Isolated Sp and YS were kept for up to 6 days in organ culture without exogenous growth factors.

When transferred into culture, the dissected YS soon resumed the initial morphology and increased in size (Figure 1). In YS explants isolated at presomite stages, red blood cells were found to develop, and clearly identifiable blood islands were observed. A network of small and large diameter blood vessels expressing the endothelial marker CD31 subsequently developed in the explants (data not shown). In most cases, the initial organization of the Sp explants was also maintained, with the hindgut, the two aortae, and caudal omphalomesenteric artery still identifiable.

Cell suspensions from individual explants were obtained at various intervals after the onset of organ culture. They were then cultured for an additional 6–12 days with stromal cells, *c-kit* ligand, and IL-7 and analyzed by flow cytometry for the presence of committed granulocytes (Gr-1), erythrocytes (TER119), and B cell (CD19) precursors.

Figure 2 shows the percentage of explants dissociated after the specified days in organ culture that gives rise to the indicated lineages after further expansion with growth factors. One hundred percent of Sp explants, kept for 4 days in organ culture, were capable of giving rise to all tested lineages. In parallel, the analysis of YS explants revealed the presence of differentiated erythrocytes and myeloid cells in all cases. Longer organ culture periods resulted in a decreased frequency of YS explants yielding myeloid cells and of Sp explants that give rise to erythroid, myeloid, and B-lymphoid cells. Given these results, we chose to use 4 days of organ culture in the subsequent experiments.

Consistent with previous observations (Cumano et al., 1996; Nishikawa et al., 1998), YS progeny never generated CD19$^+$ cells, indicating that a second generation of cells with lymphoid potential did not occur in situ (Figures 1 and 2). The addition of growth factors and stromal cells to the organ culture improved neither the frequency of positive explants nor the number of generated precursors (data not shown).

Organ Culture Alters Neither the Multipotentiality Nor the Low Levels of MHC Class I Expression in Hematopoietic Precursors Isolated before 11 Dpc

Around 2.5 $\times$ 10^3 cells were recovered from individual Sp explants and five to ten times more from YS explants (1–2 $\times$ 10^4 cells) after organ culture (Figure 1). CD45$^+$ cells, absent from freshly isolated Sp, were now readily detectable in both Sp and YS explants. These cells did not express markers of committed myeloid or lymphoid lineages. However, TER119$^+$ cells were detected in the YS, but not in Sp explants (data not shown). Clonal analysis of cells recovered from the Sp explants indicated that they contained an average of 15 B cell precursors, 80% of which also differentiated into myeloid cells, thus showing that multipotentiality was maintained.

It has previously been described that before 10.5 dpc, embryonic cells express undetectable levels of MHC class I molecules (Ozato et al., 1985; Jaffe et al., 1991). Semiquantitative RT-PCR analysis indeed showed a $>$20-fold decrease of MHC class I (K^b) expression in mRNA from sorted CD45$^+$ AGM cells compared to thymocytes (Figure 3). Complementary flow cytometric analysis revealed low to undetectable levels of D^b expression in CD45$^+$ hematopoietic cells from 11 dpc YS

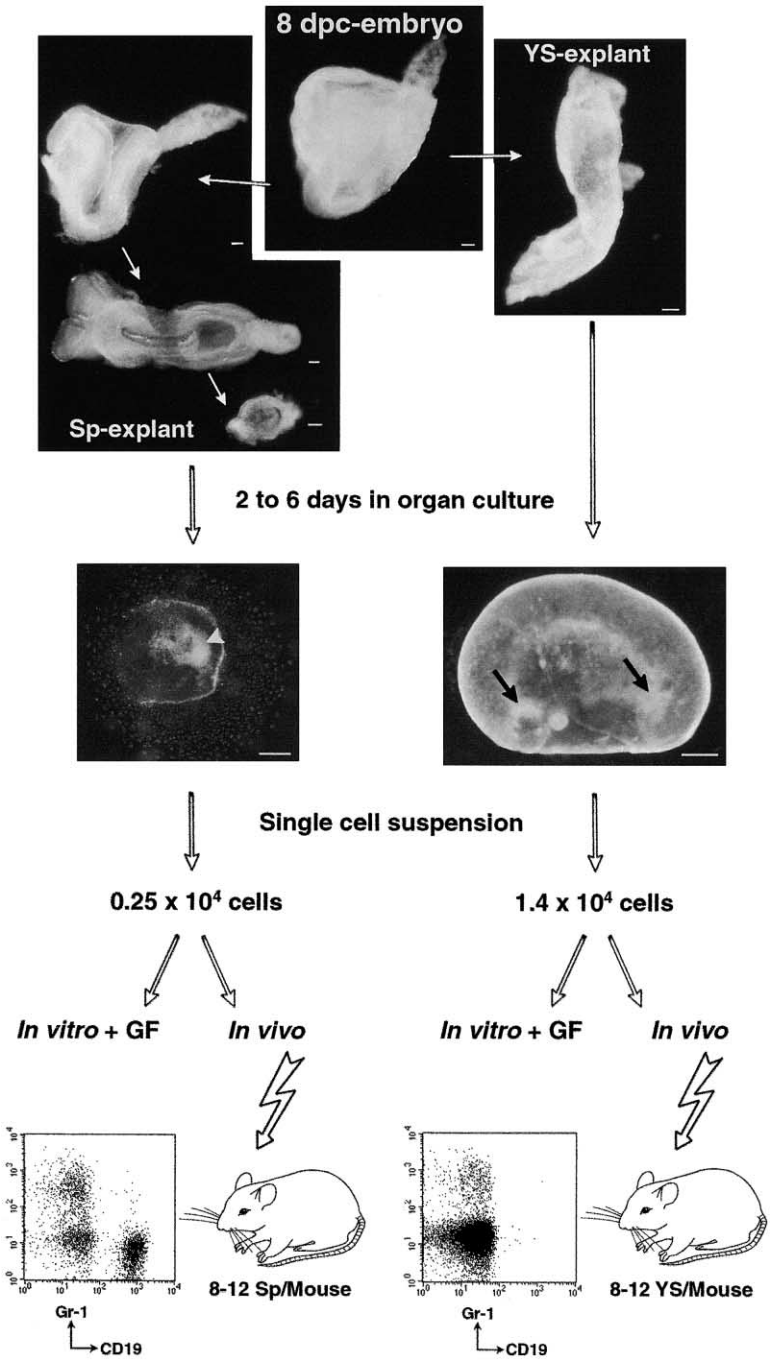

Figure 1. Experimental Strategy Used to Assess In Vivo Reconstitution Capacity of Hematopoietic Precursors Derived from Precirculation YS and Sp Explants

The YS and Sp are shown during dissection (scale bars represent 100 μm) from a 5 S embryo and after 3 days in organ culture (scale bars represent 500 μm). Patches of erythroid cells (arrows) are present in YS explants. The hindgut (arrowhead) retains its original shape in Sp explants. The mean number of viable cells recovered per explant after dissociation is shown. Cell suspensions were tested in parallel for their in vitro and in vivo differentiation potential. The erythro-myeloid and lymphoid progenies generated in vitro were monitored using the Gr-1 and CD19 surface markers, respectively. GF, exogenous growth factors.

and AGM cells as well as from cultured Sp and YS explants (data not shown). Collectively, the above described experiments indicated that the low levels of MHC class I expression described for the whole embryo also apply for hematopoietic cells from YS and Sp after 4 days of organ culture.

In Vivo Reconstitution Capacity of Embryonic Hematopoietic Cells Isolated before the Onset of Circulation

Experimental Scheme

As mentioned previously, embryonic hematopoietic cells isolated before the establishment of circulation cannot reconstitute hematopoiesis in normal adult recipients (Cumano et al., 1996). We therefore decided to use as recipients two strains of immunodeficient mice, *Rag2*$^{-/-}$ and *Rag2*γc$^{-/-}$, both available in the C57BL/6 background. The lack of an immature lymphoid compartment in these mutant mice provides a suitable environment for engraftment of lymphocyte precursors in the absence of competition. These conditions were chosen to favor the emergence of a lymphoid potential in YS cells. In addition, embryonic cells, expressing low levels of MHC class I molecules, cannot be targeted by NK cells, which are absent in *Rag2*γc$^{-/-}$ mice.

Sp and YS from C57BL/6 Ly5.1 embryos isolated be-

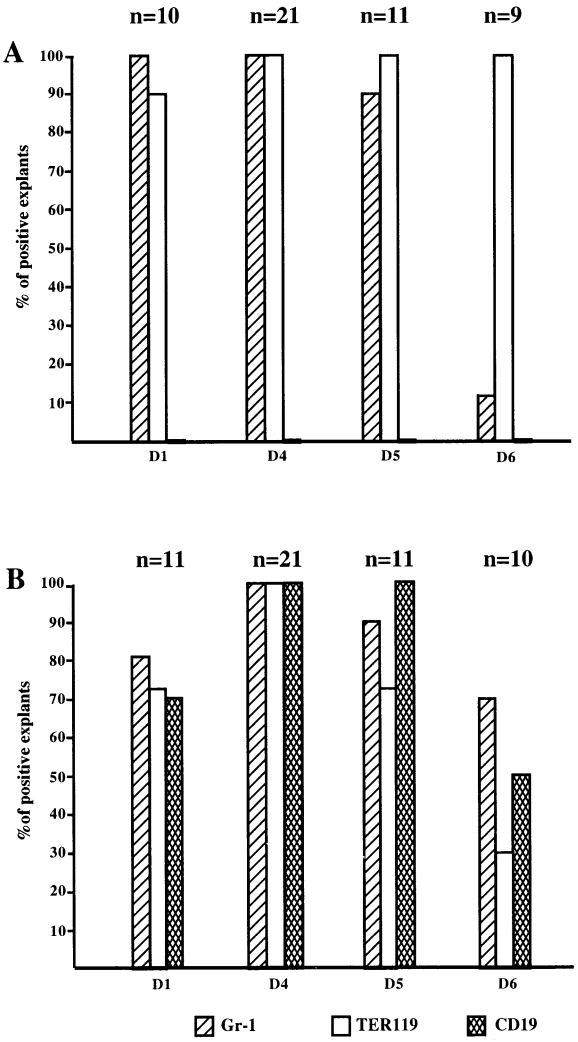

Figure 2. Hematopoietic Progeny Derived from 0–10 S YS (A) or Sp (B) at Various Time Points after Organ Culture

Explants were kept in organ culture for the periods of time mentioned on the *x* axis. Cell suspensions of individual explants were further cultured for 10–12 days (see Experimental Procedures) and analyzed by flow cytometry. Shown are the percentages of explants positive for Gr-1 (granulocytes), TER119 (erythrocytes), and CD19 (B lineage cells). n, number of embryos tested.

tween the 0 and 10 S stages (7.5–8.5 dpc) were dissociated after organ culture. Pools of cells from 8–12 Sp or YS explants were then intravenously injected into 400–600 rads of γ-irradiated *Rag2*$^{-/-}$ or *Rag2*γ*c*$^{-/-}$ recipient mice. YS-derived cells were also injected directly after dissection and are referred as YS D0. AGM cells, a source of LTRs (Müller et al., 1994), were injected into the two types of recipients as a positive control. Mice were bled 1 and 3 months after injection and sacrificed between 6 and 8 months after reconstitution.

Short-Term Reconstitution

The surface phenotype of peripheral blood cells obtained after injection was analyzed by flow cytometry. Figure 4A shows the profiles of donor-derived Ly5.1^{+} blood cells isolated from *Rag2*γ*c*$^{-/-}$ mice 1 month after injection with AGM and organ-cultured YS, and stained

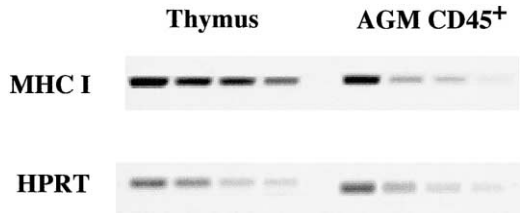

Figure 3. Levels of MHC Class I Expression in Hematopoietic Cells Isolated from CD45^{+} AGM Cells

Semiquantitative RT-PCR analysis of MHC class I expression on sorted CD45^{+} cells from 10.5–11.5 dpc AGM and on adult thymocytes. 1:3 dilutions of cDNA are shown.

for CD19 and Gr-1. Both profiles show the presence of myeloid cells, indicating that the injected precursors engrafted the adult recipients. Myeloid reconstitution following injection with YS cells was obtained in three out of seven *Rag2*γ*c*$^{-/-}$ but in none of the *Rag2*$^{-/-}$ mice. Reconstitution was not observed when YS cells were injected prior to organ culture (YS D0). This result was consistent regardless of the number (8–12 embryo equivalents) of YS cells injected (Table 1A). Three months after injection, donor-derived myeloid cells were no longer present in recipients of cultured YS cells.

In contrast, *Rag2*γ*c*$^{-/-}$ mice injected with either AGM or Sp cells contained circulating Ly5.1^{+} T and B cells 3 months after transfer (Figure 4B). All mice injected with AGM precursors (Table 1C) and the majority (6/10) of mice injected with Sp precursors (Table 1B) had circulating myeloid cells (Figure 4B).

These data show that while AGM and Sp precursors are endowed with short-term multilineage reconstitution capacity, YS cells cannot generate lymphocytes but can reconstitute the myeloid compartment of the recipient mice (although for less than 3 months). This result indicates that YS precursors can home and differentiate in the recipient bone marrow environment of adult mice.

Long-Term Reconstitution

Mice were analyzed 6–8 months after injection for the assessment of LTR activity. Figure 5 shows donor-derived (Ly5.1^{+}-gated) myeloid and lymphoid cells both in the spleen and bone marrow of *Rag2*γ*c*$^{-/-}$ mice that received Sp or AGM cells. Ly5.1^{+} cells were undetectable in mice recipients of YS-derived precursors (Figure 5; Table 1A). No myeloid cells and no B cell precursors were detected in the bone marrow of *Rag2*$^{-/-}$ recipient mice injected with Sp-derived cells, indicating that long-term reconstitution of the hematopoietic system had not occurred (Table 1B). All mice displayed donor-derived B lymphocytes in the peripheral organs and all but one also had T lymphocytes. In contrast, the four *Rag2*γ*c*$^{-/-}$ recipients injected with 10–12 embryo equivalents of Sp-derived precursors showed long-term reconstitution. This result establishes that precursors generated in the Sp can ensure long-term hematopoiesis in adult recipient mice.

AGM cells reconstituted *Rag2*γ*c*$^{-/-}$ more efficiently (4/4) than *Rag2*$^{-/-}$ (3/8) recipients (Table 1C). Moreover, the percentages of donor-derived myeloid cells in the bone marrow were consistently higher in *Rag2*γ*c*$^{-/-}$ mice. It has previously been shown that before 10.5 dpc

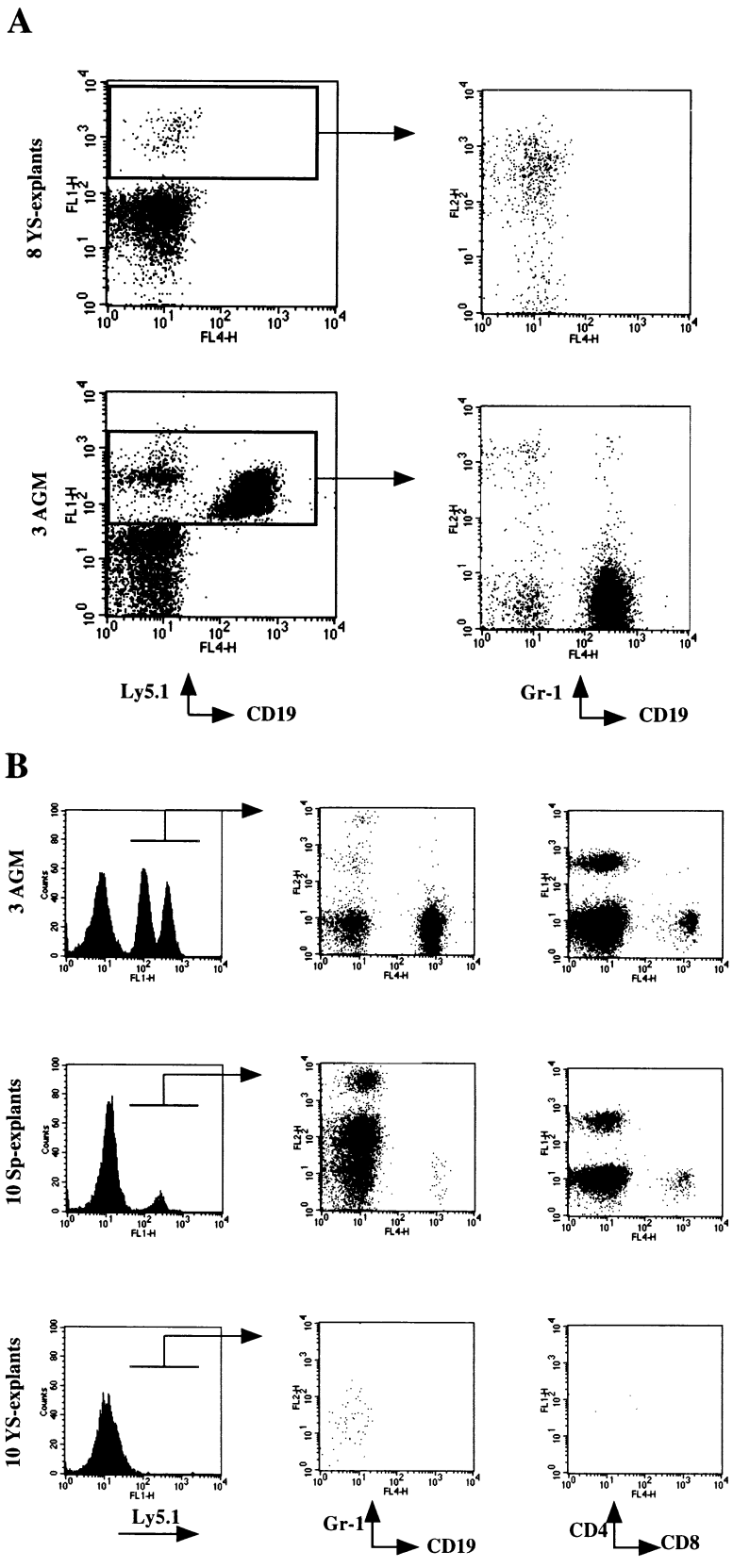

Figure 4. Analysis of Recipient Mice for Short-Term Reconstitution

Analysis of donor-derived hematopoietic progeny in the peripheral blood of recipient *Rag2*$\gamma c^{-/-}$ mice.

(A) One month after the injection of cells isolated from YS after organ culture or from 11 dpc AGM.

(B) Three months after the injection of Sp and YS cells after organ culture or of 11 dpc AGM cells. A representative example is shown for each group of recipients summarized in Table 1.

Table 1. Summary of Reconstitution Experiments

A	Short-term reconstitution (blood) B cells	T cells	Granulocytes	NK cells	Long-term reconstitution
8 YS D4 (0–10 S)/recipient 2 *Rag2*$^{-/-}$	0/2	0/2	0/2	0/2	0/2
8.5 YS D0 (0–10 S)/recipient 2 *Rag2*$^{-/-}$	0/2	0/2	0/2	0/2	0/2
8–12 YS D4 (0–10 S)/recipient 7 *Rag2*γc$^{-/-}$	0/7	0/7	3/7	0/7	0/7
8–12 YS D0 (0–10 S)/recipient 4 *Rag2*γc$^{-/-}$	0/4	0/4	0/4	0/4	0/4

B	Short-term reconstitution (blood) B cells	T cells	Granulocytes	NK cells	Long-term reconstitution (% donor-derived granulocytes in BM)
8 Sp D4 (0–10 S)/recipient 2 *Rag2*$^{-/-}$	2/2	0/2	2/2	0/2	0/2
8 Sp D4 (0–10 S)/recipient 4 *Rag2*γc$^{-/-}$	4/4	3/4	2/4	4/4	0/4
10–12 Sp D4 (0–10 S)/recipient 4 *Rag2*γc$^{-/-}$	4/4	3/4	2/4	4/4	4/4 (3%) (2%) (1%) (5%)

C	Short-term reconstitution (blood) B cells	T cells	Granulocytes	NK cells	Long-term reconstitution (% donor-derived granulocytes in BM)
3 AGM (28–32 S)/recipient 3 *Rag2*$^{-/-}$	0/3	0/3	3/3	3/3	0/3
3.5 AGM (40–45 S)/recipient 3 *Rag2*$^{-/-}$	3/3	1/3	3/3	3/3	2/3 (<1%) (73%)
8 AGM (40–45 S)/recipient 2 *Rag2*$^{-/-}$	2/2	1/2	2/2	1/2	1/2 (<1%)
3 AGM (30–35 S)/recipient 1 *Rag2*γc$^{-/-}$	1/1	1/1	1/1	1/1	1/1 (2%)
3 AGM (40–45 S)/recipient 2 *Rag2*γc$^{-/-}$	2/2	0/2	2/2	2/2	2/2 (90%) (90%)
8 AGM (40–45 S)/recipient 1 *Rag2*γc$^{-/-}$	1/1	1/1	1/1	1/1	1/1 (32%)

Short-term (1–3 months) and long-term (6–8 months) reconstitution obtained after the transfer of 7.5–8.5 dpc YS-derived (A) and Sp-derived (B) precursors after 4 days in organ culture and of 10–11.5 dpc AGM (C) into *Rag2*$^{-/-}$ (in gray) or *Rag2*γc$^{-/-}$ (in white) recipient mice. Long-term reconstitution was considered to be present when a minimum of 0.5% of donor-derived B and myeloid cells were present in the bone marrow. Shown in parentheses in (B) and (C) are the percentages of myeloid cells of donor origin found in the bone marrow of long-term reconstituted mice.

(35 S), AGM cells do not provide LTR in normal recipients (Müller et al., 1994; Ohmura et al., 1999). The same result was observed here when 9.5–10 dpc (28–32 S) AGM cells were transferred in *Rag2*$^{-/-}$ recipients; short-term reconstitution of myeloid and NK cells was observed, but not any LTR activity. In contrast, hematopoietic cells derived from 3 AGM at 9.5–10 dpc could provide LTR when transferred into a *Rag2*γc$^{-/-}$ recipient.

Discussion

Here, we show that 7.5–8 dpc Sp, but not YS-derived precursors, can provide long-term reconstitution of the hematopoietic system of adult immunodeficient mice. Hematopoietic precursors derived in vitro from the intraembryonic hemogenic site explanted before circulation was established, thus qualifying as HSC.

In the mouse, the vascular network is already fully developed when LTR activity was previously detected in the YS and P-Sp by means of a newborn transplantation assay (Yoder et al., 1997). Reconstitution could result from the transfer of hematopoietic cells originating in either extra- or intraembryonic compartments via the blood flow. To settle this issue, the LTR potential of YS- and Sp-derived hematopoietic precursors has to be analyzed prior to the establishment of vascular connections between the extra- and intraembryonic compartments.

In a transplantation protocol using normal irradiated mice as recipients, neither Sp- nor YS-derived precursors separated before circulation proved capable of either short- or long-term reconstitution. Even after direct intrasplenic injection, CFU-S activity was not detected (Cumano et al., 1996). We now chose to reconstitute *Rag2*$^{-/-}$ mice that were deprived of a lymphoid compartment due to a mutation in the *Rag2* gene required for antigen receptor somatic rearrangement (Shinkai et al., 1992). However, these mice have a normal compartment of bone marrow and thymic early lymphoid precursors. As an alternative recipient strain, we used *Rag2* x γc double mutant mice (Colucci et al., 1999). *Rag2*γc$^{-/-}$ mice are depleted of lymphoid precursors and show a complete absence of NK cell activity due to impaired

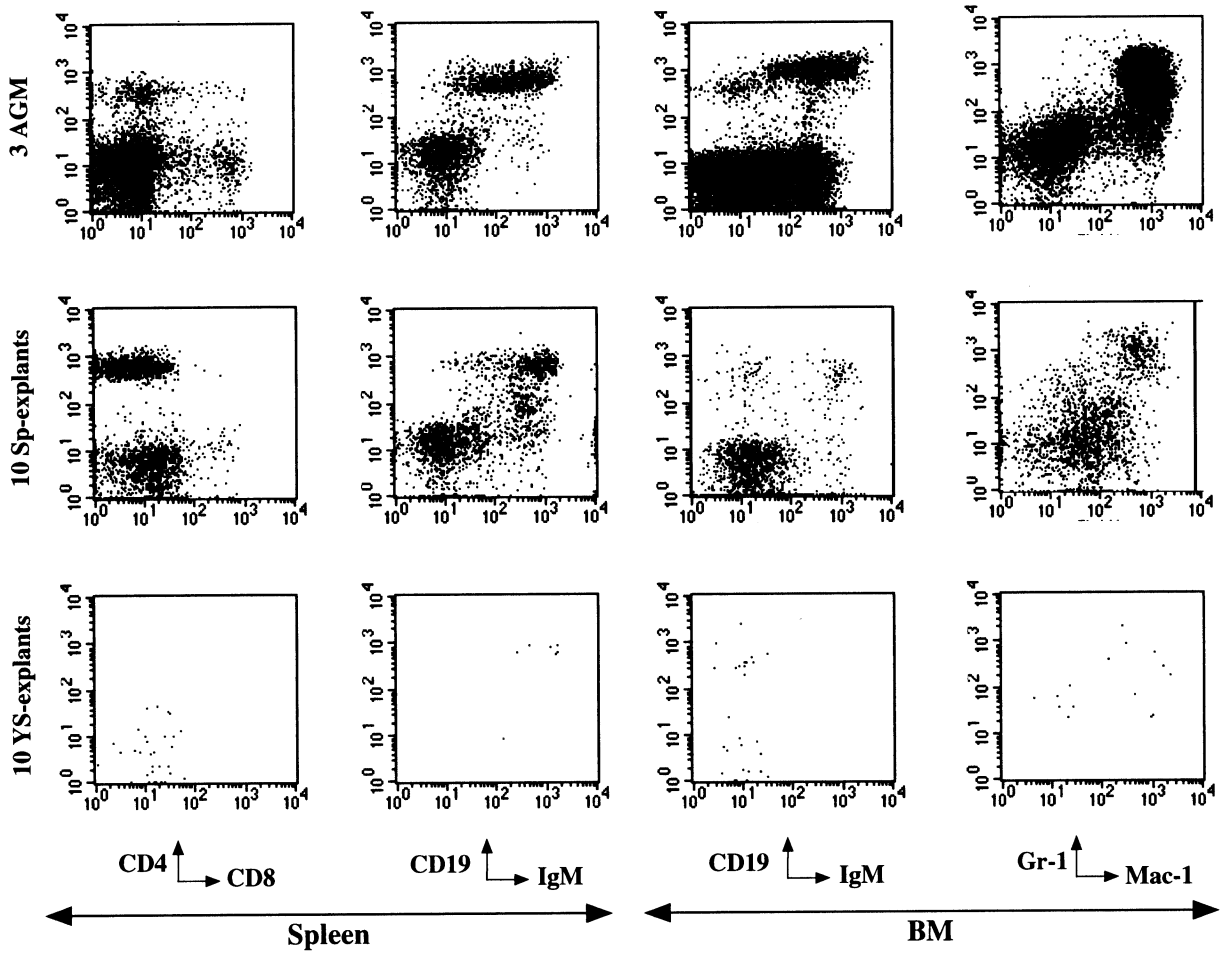

Figure 5. Analysis of Recipient Mice for Long-Term Reconstitution

Flow cytometric analysis of donor-derived hematopoietic cells in spleen and bone marrow of *Rag2*$\gamma c^{-/-}$ recipient mice, analyzed 6 to 8 months after transfer. All dot plots refer to Ly5.1-positive (donor) populations of the same recipients shown in Figure 4B.

signaling through IL-7 and IL-15 receptors. The absence of NK cells might improve the engraftment capacity due to the lack of recognition of low levels of MHC class I expression in embryonic tissues. The transfer in these two immunodeficient strains thus aimed at providing the best condition for the expression of a lymphoid potential and to prevent NK-mediated rejection.

When injected in both mutant recipients, AGM and cultured Sp-derived progenitors readily gave rise to both B and T lymphocytes that could still be found 8 months after transplantation. However, *Rag2*$^{-/-}$ mice that received Sp-derived cells were not long-term reconstituted, since we failed to detect both myeloid and lymphoid donor-derived cells in the bone marrow at the latest time points of analysis. In contrast, *Rag2*$\gamma c^{-/-}$ injected with this same population contained both cell lineages in the bone marrow and in the peripheral organs 8 months after transplantation, therefore showing long-term reconstitution.

The organ culture step allowed a continuous generation of hematopoietic precursors in the Sp. These precursors are similar in many respects to those found in the 10.5–11.5 dpc AGM: (1) CD45^{+} cells from cultured Sp explants do not include precursors expressing significant levels of mRNA or cell surface markers that characterize committed myeloid or lymphoid cells. We previously showed that the AGM environment, while generating multipotent precursors, does not allow for their in situ differentiation (Godin et al., 1999); (2) Sp precursors, like those from the AGM, are multipotent, since they can give rise in vitro to erythro-myeloid as well as lymphoid cells in the presence of stromal cells and appropriate growth factors; (3) we establish here that CD45^{+} cells from both explants express low levels of MHC class I, thus extending to early hematopoietic cells the observation already made for the whole embryo (Ozato et al., 1985; Jaffe et al., 1991), 9 dpc P-Sp (Yoder et al., 1997), and 11 dpc YS (Huang and Auerbach, 1993); and (4) finally, cultured Sp and AGM precursors manifest their ability to reconstitute, in the long term, all hematopoietic lineages when transplanted into adult immunodeficient mice, indicating that Sp organ culture can sustain the generation of HSC. We conclude that during mouse embryogenesis, HSCs are generated in the intraembryonic Sp/AGM region.

Whereas HSCs derived from cultured Sp qualitatively resemble AGM HSCs, limited dilution analysis established that the number of multipotent precursors only

represents about 15% of that found in the 10.5–11.5 dpc AGM (about 100 per AGM; Godin et al., 1999). This quantitative difference most probably accounts for the need to inject three to four times more Sp than AGM cells in order to obtain LTR.

Previous experiments established that the long-term repopulating activity of AGM, as assayed by transplantation into conventional irradiated mice, could only be detected after 10.5 dpc (35–40 S stage; Müller et al., 1994; Ohmura et al., 1999). *Rag2*$\gamma c^{-/-}$ used as recipients allowed the demonstration of such a potential at earlier stages (30–35 S). Cell populations that were previously shown to be incapable of LTR activity in adult mice such as AGM cells before the 35 S stage and 8 dpc Sp maintained in organ culture showed LTR potential when transplanted into *Rag2*$\gamma c^{-/-}$ but not *Rag2*$^{-/-}$ recipients. The improved engraftment capacity in the former, not only apparent for lymphoid but also for myeloid cells, indicates that the absence of NK cell activity might indeed be an important factor in this process. This observation could have therapeutic implications when embryonic tissues are to be used in transplantation protocols.

Rag2$^{-/-}$ mice injected with YS cells after organ culture or directly ex vivo showed an absence of donor-derived cells at all time points tested. *Rag2*$\gamma c^{-/-}$ mice injected with YS cells after organ culture showed myeloid reconstitution 1 month after transfer, indicating that cells could home to the bone marrow and generate a mature progeny. This reconstitution was transient, and 3 months after transfer, donor-derived progeny were no longer observed in the blood. No lymphocytes were ever detected in these mice even when ten or twelve embryo equivalents were injected. These results indicate that hematopoietic cells of YS origin, dissected before the establishment of circulation, do not have a lymphoid differentiation potential. Moreover, a 4 day organ culture also failed to reveal such a potential, a result that does not support the hypothesis of a second generation of LTR-endowed HSC in the YS. The differentiation potential of mammalian YS cells thus differs from that of *Xenopus* ventral blood islands (the YS equivalent), which contribute to T cell generation (Bechtold et al., 1992). As all of the attempts to derive lymphoid cells from YS precursors before circulation, either in vitro or in vivo, consistently failed, YS hematopoietic cell generation appears restricted to erythro-myeloid cells for a short period of embryonic development.

Experimental Procedures

Animals and Dissection of Embryonic Structures

The two C57BL/6 congenic lines bearing the Ly5.2 and Ly5.1 alleles of the pan-hematopoietic marker CD45 and the F1 progeny of these mice were used in the course of this study. Two strains of immunodeficient mice, *Rag2*$^{-/-}$ (Shinkai et al., 1992) and *Rag2*$\gamma c^{-/-}$ (Colucci et al., 1999), both carrying the Ly5.2 allele, were used as recipients in the reconstitution experiments. Reconstituted mice were housed throughout the experiments in sterile ventilated cupboards at the Pasteur Institute animal facilities.

The day of vaginal plug observation was considered as day 0.5 postcoitum. Pregnant females between 7.5 and 11.5 dpc were sacrificed by cervical dislocation. Each embryo was staged by somite counting or, during presomite stages, according to the criteria of Downs and Davies (1993). Dissections of 7.5–8.5 dpc Sp and YS, and of 10.5–11.5 dpc AGM were carried out as previously described (Cumano et al., 1996; Godin et al., 1999).

Culture Conditions

Organ Culture

YS and Sp explants were individually transferred into wells of a 24 well plate containing culture medium (OptiMEM, GIBCO) supplemented with 10% fetal calf serum and 5×10^{-5} M 2-ME and cultured in toto for up to 6 days. At various intervals during the culture period, single-cell suspensions were prepared from individual explants by passage of the selected tissues through a 26 gauge needle.

Hematopoietic Progeny Assay

Cell suspensions from Sp or YS explants were further cultured for 6–12 days on γ-irradiated S17 stromal cells (a kind gift of K. Dorshkind, Riverside, CA) as previously described (Cumano and Paige, 1992; Cumano et al., 1992). The following cytokines were added: interleukin 7 (IL-7) at 50–100 units per ml, provided by the supernatant of a stably transfected cell line (from Fritz Melchers, Basel) and *c-kit* ligand (KL; Genetics Institute) at a 1:50 dilution, which allows the emergence of mast cells from adult bone marrow.

Gene Expression Analysis

Total RNA was isolated from freshly dissected 10.5–11.5 dpc AGM and from thymocytes using TRIzol (GIBCO-BRL), according to the manufacturer's protocol. RNA extracted from adult spleen and thymus was used as a positive control. Oligo(dT)-primed cDNA was prepared from total RNA derived from 5×10^5 to 2×10^7 cells using avian myeloblastosis virus reverse transcriptase (RT) in a reaction volume of 20 μl under conditions recommended by the manufacturer (GIBCO-BRL). cDNA was sequentially diluted at 1:3. To compensate for variable RNA and cDNA yields, the amount of cDNA synthesized was calibrated using the relative expression level of hypoxanthine phosphoribosyltransferase (HPRT) as a standard. PCR was performed with primers specific to MHC class I (K^b) and HPRT (Douagi et al., 2000; Li et al., 1993) in 50 μl samples using 1 unit Taq polymerase, and was carried out in a thermal cycler (Perkin Elmer) under the following conditions: 94°C for 30 s, 55°C–60°C for 30 s, and 72°C for 30 s, for 30 cycles. Ten microliters of the resulting amplified material was fragmented on 1.8% agarose gels in TBE and stained with EtBr. All PCR products were within the exponential phase, allowing the comparison of band intensity between samples. Scanned gels were processed using the Graphic Converter software.

Reconstitution Experiments

Eight dpc Sp and YS explanted from F1 embryos (C57BL/6 Ly5.1 x Ly5.2) were cultured in toto for 4 days. Cells collected from pooled explants were injected in the retroorbital sinus of sublethally irradiated (400–600 rad) *Rag2*$^{-/-}$ or *Rag2*$\gamma c^{-/-}$ recipient mice. Cells from the AGM region were injected immediately after dissection from 28–45 S embryos. Eight dpc YS were also injected upon explantation.

For short-term reconstitution analyses, peripheral blood was collected from recipient mice at 1 to 2 month intervals and analyzed by flow cytometry for the expression of donor-derived (Ly5.1), B (CD19), T (CD4 and CD8), and myeloid (Gr-1) cells. In long-term reconstitution studies, the recipient mice were sacrificed after 6 to 8 months, and the bone marrow, spleen, and thymus when present were analyzed by flow cytometry.

Flow Cytometry Analysis

Flow cytometry analysis was performed in a FACScalibur with the Cellquest program (Becton Dickinson). The Ly5 alleles were characterized using biotinylated, PE-, or FITC-conjugated antibodies 104.2 (anti-Ly5.2) or A20.17 (anti-Ly5.1). The following antibodies were used to label B and T lymphocytes: FITC-conjugated anti-IgM, PE-conjugated anti-CD4 (L3T4), APC-conjugated anti-CD45R/B220 (clone RA3-6B2), FITC-conjugated anti-CD8 (Ly-2), and biotinylated anti-CD19. PE-conjugated-Gr-1, APC-conjugated anti-Mac-1, and biotinylated-TER-119 were used to characterize cells from myeloid and erythroid lineages, respectively. Biotinylated, PE-, or FITC-conjugated anti-NK1.1 were used to identify NK cells. APC or cychrome-conjugated streptavidin were used as second step reagents. All antibodies were from Pharmingen. In all analyses, propidium iodide was used to exclude dead cells.

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