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Blood-borne seeding by hematopoietic and endothelial precursors from the allantois

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ABSTRACT **Until now the allantois has not been considered as a hematopoietic organ. Here we report experimental evidence demonstrating the *in situ* emergence of both hematopoietic and endothelial precursors in the avian allantoic bud. When the prevascularized allantoic bud from a quail embryo was grafted in the coelom of a chicken host, hematopoietic and endothelial cells later were found in the bone marrow of the host. Because the graft was located at a distance from the limb bud, these cells could reach the bone marrow only by the circulatory pathway. This blood-borne seeding may be accomplished by distinct hematopoietic and endothelial precursors, or by hemangioblasts, the postulated common precursors of these two lineages; we consider the latter interpretation more likely. We also show by reverse transcription–PCR that the allantois region expresses very early the *GATA* genes involved in hematopoiesis and some β-globin chain genes.**

Hematopoietic stem cells (HSC), which have a central role in blood renewal during vertebrate life, do not derive from any of the lineages that give rise to the stroma of hematopoietic organs (1). Instead they colonize the organ rudiments during embryogenesis. This developmental trait makes the quest for their origin compelling. HSC, once thought to become determined in the yolk sac (2, 3), later were demonstrated in birds to derive from the embryo proper, and the para-aortic mesoderm was shown to be capable of producing them (4, 5). Two groups (6, 7), revisiting this question in mammmalian embryos, reached similar conclusions. Here we demonstrate that, in the avian embryo, the allantois also has the capacity to produce HSC and thus is one of the few structures with a primordial ontogenic role in hematopoiesis.

The avian allantois is an endodermal and mesodermal embryonic appendage. It develops from a bud emitted by the posterior intestinal portal (PIP), at a time when the yolk sac has been active in erythropoiesis for about 2 days and already has released primitive erythroblasts and erythrocytes. During embryonic development the allantois is destined to fuse with another extraembryonic membrane, the chorion, to fulfill its excretory and respiratory functions (8). In mammalian embryos, after this event, the allantois contributes to the umbilical stalk and the embryonic layer of the placenta. The fusion occurs at a precise stage of allantoic development (9) and is mediated by adhesion molecules: in mouse embryos with a vascular cell adhesion molecule or an α_4-integrin knockout this fusion fails to occur (10, 11). Previous results obtained in our group on avian "yolk sac chimeras" (12) and observations by Downs and coworkers on the mouse embryo (9, 13) suggested that the allantois may have a hematopoietic function. Here we present evidence that this appendage begins producing red cells at a prevascularization stage, and when grafted from a quail into a chicken embryo contributes to the hematopoietic system of the host by colonizing the bone marrow. The graft-derived cells are hematopoietic cells and endothelial cells (EC). Finally, the expression profiles in the early chicken embryo of *GATA-1* and *GATA-3* genes, which are involved in hematopoiesis, are unique when compared with yolk sac and blood.

MATERIALS AND METHODS

Embryos and Cell Lines. Quail (*Coturnix coturnix japonica*) and chicken (JA57 strain) were used in all of the experiments. Eggs were incubated at 38°C ± 1°C and stages were determined according to the Hamburger and Hamilton (HH) nomenclature (14) for both species. Primary cells and hematopoietic cell lines, used as positive and negative controls for PCR, were chicken embryo fibroblasts, MSB1 T lymphoma cells transformed by Marek's disease virus (15), and HD11, a macrophage cell line transformed by avian myelocytomatosis virus (16). They were cultured at 37°C in DMEM supplemented with 10% fetal calf serum (Eurobio, Paris), 1% chicken serum (GIBCO), 1% penicillin-streptomycin, and 1% glutamine (GIBCO).

***In Ovo* Injections.** Five chicken embryos from stage HH16 to 20 were injected in the heart with India ink (Pelican) diluted one-fourth in PBS. The ink was inoculated into the heart by using borosilicated glass micropipettes pulled with an automated puller to obtain a tip approximately 50 μm in diameter. The tip of the pipette was introduced in the heart ventricle, and 10 μl of India ink was gently flushed into the cavity. The blood of the embryo could be completely replaced by the ink solution, if a small injury was performed in one of the large vitelline veins. Embryos were fixed overnight in 4% paraformaldehyde in phosphate buffer 0.12 M at 4°C, washed in PBS, and photographed.

Grafting Experiments. Eggs were incubated up to stages HH16 to 18 on embryonic day 3 (E3) of incubation. Either the rim of the PIP (stage HH16 donors) or the allantoic rudiment (stages HH17 and 18) were grafted from a quail donor into the coelom of a stage HH18 chicken host. Engrafted embryos, which were sacrificed at E4–6, were treated for immunohistology. Ten engrafted embryos were sacrificed at E13, at the time when the bone marrow begins differentiating, seven embryos were sacrificed at E15 and two embryos at E16 (Table 1); in all of these cases the graft with surrounding tissues as well as host leg bones was dissected. Grafts and E13 leg bones were

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Abbreviations: E, embryonic day; EC, endothelial cells; HH, Hamburger and Hamilton chicken embryo staging; HSC, hematopoietic stem cells; PIP, posterior intestinal portal; RT-PCR, reverse transcription–PCR.
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Table 1. QH1$^+$ cells in the bone marrow of chicken embryos engrafted with quail allantois

	Age of host at sacrifice	
	E15	E16
Allantois-grafted*	3.9 ± 1.8† (7)	7 ± 1 (2)
Quail control (E14)	74.1 ± 17 (2)	
Chicken control	0.33 ± 0.25 (3)	

( ) number of cases.
*Ten engrafted chicken embryos were sacrificed at E13, their bone marrow was fixed and processed for immunocytology (see text).
†% of QH1$^+$ cells ± SD; flow cytometry analysis.

treated for immunohistology. Cells from the bone marrow of E15-E16 leg bones were isolated and analyzed by flow cytometry.

Immunohistology. At early stages graft-bearing embryos were fixed whole, and later grafts were dissected as well as host leg bones. All were fixed in Bouin's solution, dehydrated, embedded in Paraplast (Sigma), and cut into 5-μm sections. Sections were rehydrated, treated 30 min in PBS supplemented with 3% fetal calf serum (GIBCO), and stained with QH1 mAb (17) followed by a 1-hr incubation with anti-mouse IgG alkaline phosphatase-conjugated antibody (Biosys, Compiègne, France). Antibody binding was detected by the alkaline phosphatase Fast Red TR/Naphtol AS/MX kit (Sigma). Sections were mounted with Aquatex (Merck) and observed in a Nikon microscope.

Flow Cytometry Analysis. E15–16 bone marrow cells were collected from each host embryo by flushing the tibias and femurs with culture medium (DMEM supplemented with 3% fetal calf serum, 1% chicken serum, 1% penicillin-streptomycin, and 1% glutamine) and passing the suspension through a 22-gauge needle. Cells from each embryo were pooled and suspensions were incubated 30 min with QH1 mAb followed by a 30-min incubation with anti-mouse IgG fluorescein isothiocyanate-conjugated antibody (Biosys) diluted 1:100 in PBS. The cells were washed three times for 10 min with culture medium between incubations. Cells were resuspended in PBS before flow cytometry analysis. Chicken and quail bone marrow cells of the same stage were analyzed as controls.

Blood Cell Collection. Blood was collected by intracardiac puncture from E3, E4, and E5 embryos. For each day, blood was pooled from 30–40 embryos. Red cells were separated by centrifugation on Histopaque-1077 (Sigma) at 2,000 t/min, 4°C during 20 min, and washed in PBS supplemented with 2% fetal bovine serum (GIBCO) before lysis.

Reverse Transcription–PCR (RT-PCR). Semiquantitative RT-PCR was performed according to standard procedures on E2.5–E5 chicken allantois and yolk sacs and E3–E5 blood cells. Allantois and yolk sac samples were collected from individual embryos (a minimum of two, a maximum of 10 embryos were analyzed), washed twice in PBS, and lysed directly. In the case of blood, two batches were analyzed for each day. Primary chicken embryo fibroblasts cells and cell lines were used as controls for our primers. RNA was extracted according to the simplified Chomczcynski method (18). Briefly, the RT step was performed for 1 hr at 37°C (Stratagene, RT-PCR kit). According to the recommendations of the supplier, 19 μl of extracted RNA were added to a master mix constituted of 0.15 ng oligo(dT) primer 100 ng/ml, 0.04 units of RNase Block Ribonuclease Inhibitor (40 units/ml), 0.05 units of Moloney murine leukemia virus-RT (50 units/ml), 0.2 mM 2′-deoxynucleoside 5′-triphosphate (4 × 25 mM each), and 5 μl of 10× first-strand buffer (Stratagene) to a final volume of 29.5 μl. PCR was performed with an Idaho Technology Rapid Cycler Machine (Idaho Falls) in a total volume of 10 μl. cDNA (0.5 to 2 μl) obtained from RT was incorporated in a master mix composed of 0.2 mM 2′-deoxynucleoside 5′-triphosphate, 0.5 mM each primer, 1.5 mM MgCl2 (50 mM, Eurobio), 1 × 10× buffer (Eurobio), 1 unit of *Taq* Extra Pol II (5 units/ml, Eurobio), 1 × 10× sucrose and cresol red, 250 mg/ml BSA (Idaho Technology), and apyrogenic water. PCR was carried out for 5 sec at 94°C, for 10 sec at 51°C, 56°C, or 58°C depending on the primers (see Table 2), and 15 sec at 72°C. This cycle was repeated 25–30 times depending on the quantity of material available. S17 was used as an internal standard.

RESULTS

Erythroid Cells Are Visible in the Allantoic Bud Before Development of the Vascular Network. Microangiographies were performed to reveal the establishment of the vascular network between the embryo and the allantois. India ink invaded the whole vascular tree, revealing its state of differentiation. In embryos injected with ink at stages with 37–40 pairs of somites (stages HH17–19), the allantois appeared unvascularized although it was surrounded by an ink-permeable endothelial network in the rim of the PIP (Fig. 1*A*). Red cells already were clearly visible in the bud. They accumulated at the apex of the allantoic appendage, away from the connection to the PIP. The allantoic bud first became ink-infiltrated 3–6 hr later between stages HH19 and 20 in embryos with 40–43 pairs of somites, the ink then obscuring red cells (Fig. 1*B*). The presence of red cells in the allantoic bud, before blood vessel development, suggests a inherent capacity of the allantoic bud to produce hematopoietic cells.

Table 2. PCR oligonucleotides

Primer	AT*	Sequence 5′-3′	Nucleotides
GATA 1 (35)	56°C	(s) CTACT GCCAC TCAGC AGCGG G	(323-343)
		(as) TTCTG GCCGT TGAGG CGGTG G	(471-451)
GATA 2 (27)	51°C	(s) CTTAC GTGCC GGCTG CCCAT G	(1122-1142)
		(as) CCGGT GCCGT CTCTT CTCCA G	(1302-1282)
GATA 3 (27)	51°C	(s) CCACC TCCTC CGCTC ATCAC C	(818-838)
		(as) GCCCG GTGCC GTCTC TTCTC C	(1030-1010)
ε globin (personal data)	58°C	(s) CAAAG TCAAC GTGGA GGAAT GC	(96-117)
		(as) CTGCA GCTCA GTGGT ACTTG CG	(475-496)
ρ globin (personal data)	58°C	(s) AAAGT CAACG TGGAG GAATG C	(97-117)
		(as) TGGGA GCTCA GTGGT ACTTG TA	(475-496)
β globin (36)	58°C	(s) TCTCC CAACT GTCCG AAC	(308-325)
		(as) TAGGT GCTCC GTGAT CTT	(508-525)
S17 (37)	51–58°C	(s) TACAC CCGTC TGGGC AACGA C	(61-81)
		(as) CCGCT GGATG CGCTT CATCA G	(189-169)

(s), sense. (as), antisense.
*Annealing temperature. S17 annealing temperature was matched to that for the GATA or globin primers in use.

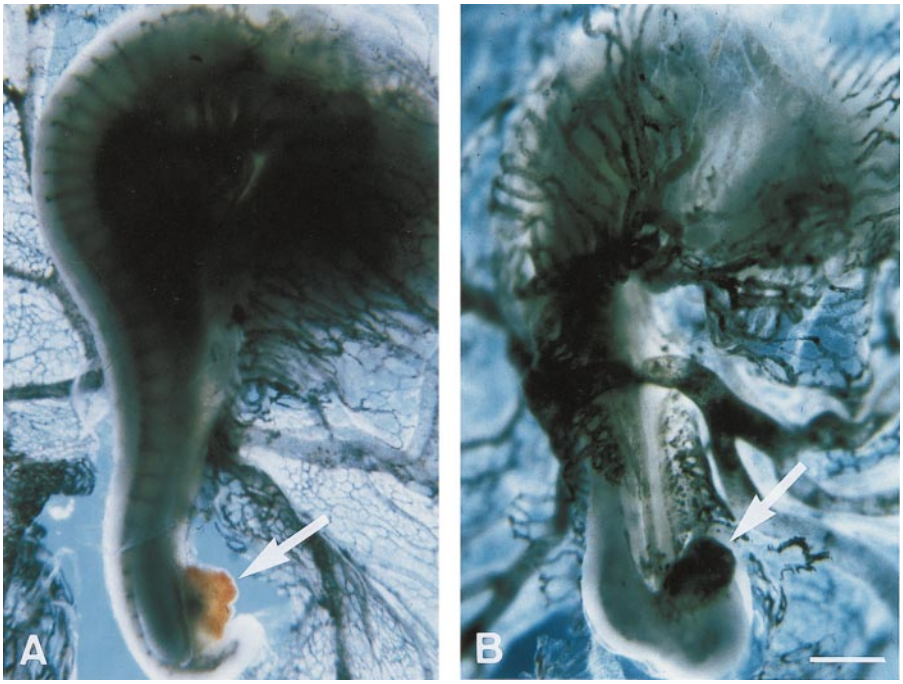

FIG. 1. Chicken embryos injected with India ink in the heart. (*A*) At 72 hr of incubation (stage HH 18) the ink did not permeate the allantoic bud (arrow), which appeared red with erythrocytes or erythroblasts. (*B*) At 84 hr, ink infiltration masked red cells caused by establishment of vascular connections. (Bar = 0.5 mm.)

An Intrinsic Endothelial Network Develops in Grafted Quail Allantoic Buds. To establish that HSC become committed from the allantoic mesoderm, quail to chicken grafting experiments were implemented. The origin of blood vessels and hematopoietic cells in the grafts was determined by means of the QH1 mAb whose affinity is restricted to quail EC and hematopoietic cells; erythrocytes lose this affinity (17). To avoid the presence of hematopoietic cells carried into the graft by the blood, the rudiments were obtained from very early embryos, namely stages HH16–17, when the allantois was a very small unvascularized bud or even earlier when the bud had not yet evaginated from the PIP. In this case the rim of the PIP, including the presumptive allantoic territory, was grafted. In the coelom environment, the quail graft usually developed as a vesicle attached to the host liver or lung by means of vascular connections. In all cases the graft developed a $QH1^+$ endothelial network and usually became a inflated red ball, full of erythroid cells. $QH1^+$ blood island-like formations sometimes were observed. In embryos sacrificed 1–3 days after engraftment, EC from the graft invaded the host surrounding tissues, making up all-quail tubes that merged progressively with chimeric tubes; occasional $QH1^+$ blood island-like structures were even present in the host tissues (data not shown).

Allantoic Graft-Derived Hematopoietic Cells and EC Colonize the Host Bone Marrow. In 10 engrafted chicken hosts examined at E13, $QH1^+$ hematopoietic cells, $QH1^+$ EC, and in some cases $QH1^+$ osteoclasts (data not shown), were found in the host bone marrow (Fig. 2). In some embryos the amount of hematopoietic cells of quail origin was impressive (Fig. 2 *C* or *D*). To obtain quantitative data concerning this colonization process, bone marrow from older embryos was submitted to flow cytometry analysis using QH1 as a probe. $QH1^+$ cells were detected in all of the E15 and E16 experimental samples (Table 1) and, while contributing a mean of 4.6% of host bone marrow cells, constituted as much as 8% in some embryos (Fig. 3).

Expression of Hematopoietic Genes in the Early Allantois. It seemed interesting to compare hematopoietic-specific gene expression in the early allantois (E2–5 to E5) with this expression in the yolk sac and blood, as erythropoiesis is active in the latter sites at this time. The expression profiles of *GATA-1*, *GATA-2*, and *GATA-3* genes as well as of ρ and ε primitive and β definitive globin genes were analyzed in cells from the three origins by RT-PCR (Fig. 4). Blood, which had to be pooled from 30 embryos, was examined only from E3 onward. Organs from individual embryos were used for mRNA extraction. The amount of each mRNA in individual organs permitted only a semiquantitative analysis, with S17 mRNA as an internal control. Unexpectedly it proved difficult to find nonexpressing tissues as controls. Primary fibroblasts and immortalized cell lines, belonging to the myeloid and lymphoid lineages, which did not express any of the globin genes studied, served as controls for the RT-PCR procedure. The MSB1 cell line expressed *GATA-3*, as previously established by Leonard *et al.* (19). Interestingly, a highly reproducible expression pattern of GATA factors, unique to the allantois in comparison with yolk sac and blood, was found. The most striking specificity concerned *GATA-3*, which was expressed in the allantois from E3 (around stage HH 18) onward, although it never was expressed in the yolk sac nor in the blood. *GATA-1* was restricted to the earliest stages examined in the allantois (E2.5 and E3) and disappeared at E4; in contrast it was present in the yolk sac and blood cells in the period considered; *GATA-2* was expressed in all three tissues at all stages analyzed. The globin expression profiles were identical in blood cells and yolk sac cells. In the allantois ρ, ε, and β major mRNAs were detected slightly later, usually from stage HH 18 (E3) onward, and persisted at E5, the last stage studied.

DISCUSSION

These experiments in the avian model yield three conclusions; (*i*) the allantois acquires its endothelial network through vasculogenesis; (*ii*) the allantois produces HSC, which are capable of colonizing the embryo's bone marrow; and (*iii*) EC or EC precursors from the allantois, capable of migrating a long distance via the circulation, might have an important role in the development of the bone marrow.

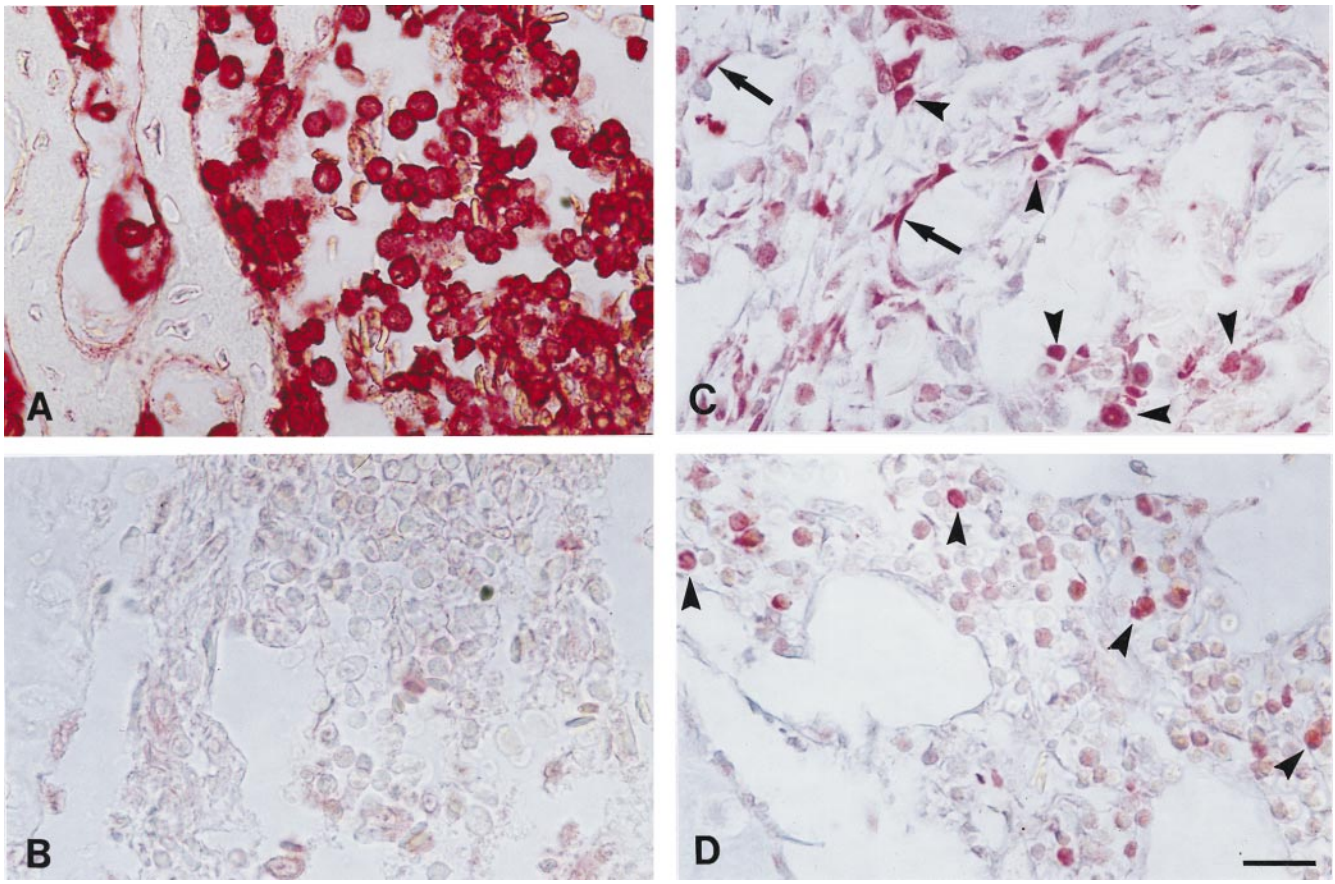

FIG. 2. QH1 immunostaining of E13 bone marrow. (*A*) Control quail, (*B*) control chicken, and (*C* and *D*) chicken embryos engrafted with quail allantois. EC (arrows, *C*) and hematopoietic cells (arrowheads in *C* and *D*) sometimes were distributed in distinct areas. (Bar = 15 μm.)

Vasculogenesis, or *in situ* emergence of EC, is one of the mechanisms of EC differentiation during ontogeny. It occurs characteristically in the whole splanchnopleural mesoderm, which also is responsible for HSC production as demonstrated in the quail-chicken system by transplanting either the E1.5 splanchnopleural mesoderm or E3 visceral organ rudiments (20, 21). EC, which differentiated from the transplanted mesoderm, vascularized the transplants entirely, whichever the stage of isolation. Angiogenesis, the other process recognized to account for EC network differentiation in the embryo, involves the migration of EC precursors of somitic origin, which seed the somatopleural mesoderm of the lateral plate (22). In this process, the EC precursors, which precede myoblasts (23), migrate through an interstitial pathway.

The occurrence of vasculogenesis and HSC emergence in the allantois is thus a relevant instance of the general rule, according to which these processes occur in endoderm-associated mesoderm. The transplantation experiments, which allowed this rule to be established, all were carried out after the gastrulation movements, during which mesodermal cells invaginate through the primitive streak and move out centrifugally. After these migrations, when the mesoderm splits into somatopleural and splanchnopleural layers, only the latter produces EC. The allantois bud is an emanation of the

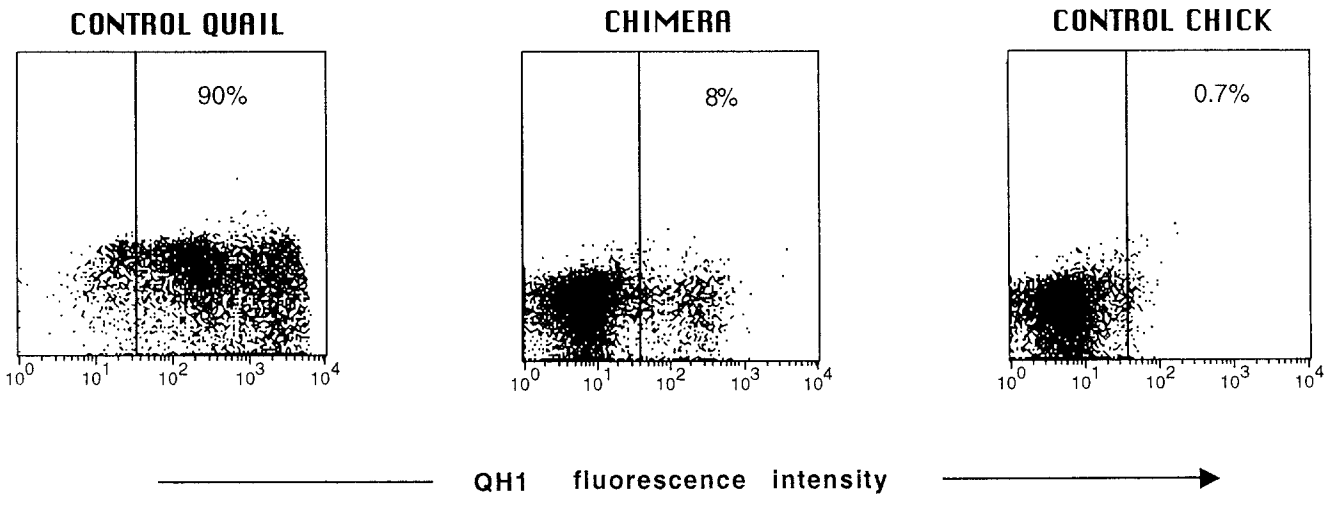

FIG. 3. Bone marrow cell flow cytometry with QH1. Control quail, E14. Chimeras, E15. Control chicken, E15.

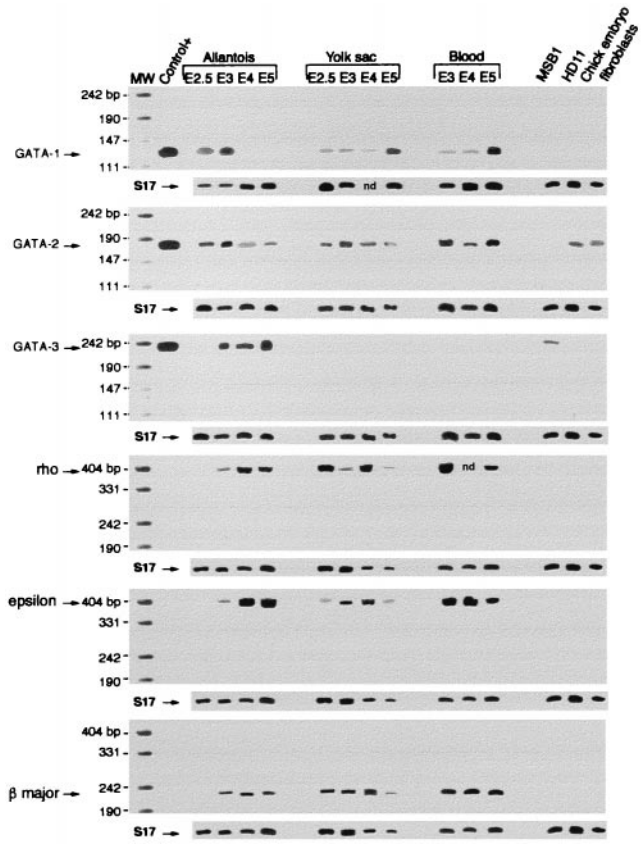

FIG. 4. RT-PCR early developmental expression of GATA factors and globins in chicken allantois, yolk sac, and blood. Blood was pooled from about 30 embryos for each day; allantois and yolk sac cells were collected from individual embryos. Control +, plasmids containing *GATA* cDNAs. nd, not determined.

splanchnopleura, thus it fits with the expectation that EC proceed from intrinsic precursors, like in other splanchnopleural derivatives.

In contrast to the emergence of EC, the emergence of hematopoietic progenitors, while it also occurs in the early splanchnopleural mesoderm, becomes restricted within the embryo to the paraaortic region, when visceral rudiments undergo organogenesis. By virtue of this developmentally regulated dissociation between the hematopoietic and angiogenic potentials, it has been possible to experimentally dissect the endothelial network from the HSC, by transplanting visceral organ rudiments, for instance the spleen (20).

The allantois retrieved as a presumptive territory (i.e., a fragment of the splanchnopleura) or as a bud produced HSC, thus this appendage is one of the very few primordial hematopoietic organs. India ink injections allowed us to establish the stage when patent vessels appear in the allantoic bud, so that we could be sure that no blood-borne HSC had seeded the bud before transplantation. The allantois now must be added to the list of sites recognized to produce HSC, which consisted up to now in the yolk sac and the para-aortic region. Therefore the capacity to produce HSC appears extensively distributed in mesoderm of the embryo and extraembryonic appendages, where it seems specified by two conditions; as mentioned above association with endoderm, a germ layer hypothesized to produce appropriate inductive signals (24), and nonengagement into visceral organ differentiation. The association with endoderm may be short-termed, as in the case of the paraaortic region.

Earlier data on yolk sac chimeras led our group to attribute an important role to the para-aortic region in definitive hematopoiesis (25, 26). These chimeras consisted of a quail embryo grafted on a chicken yolk sac. The entirely quail constitution of thymus, bursa of Fabricius, and bone marrow as well as the progressive circulatory release of quail red cells in these chimeras indicated that HSC were produced by the embryo proper, wherein the para-aortic region could be shown to produce HSC (25, 26). Blood-island-like aggregates sometimes were observed in the allantois of these chimeras (12). The present data ascertain that the presence of these aggregates is linked to the emergence of HSC. The interesting implication is that the allantois, an emanation of the grafted quail embryo in the yolk sac chimera model, contributed to the seeding of definitive hematopoietic organ rudiments.

By comparison with the yolk sac, initiation of hematopoiesis is delayed in the allantois, because the allantoic bud is emitted in the avian embryo when yolk sac erythropoiesis is already full blown. Interestingly at identical stages, *GATA-1* and *GATA-3* gene expression profiles are different, suggesting specific hematopoietic programs in each of these organs. Data about ontogenic expression of these avian transcription factors are scarce. *GATA-1* is expressed in erythroid cells apparently in relation to β-like globin chain ε production (19, 27), whereas *GATA-3*, expressed in neural tissue and ectoderm, was found restricted in hematopoietic tissues to T lymphocytes and definitive erythroid cells (27). *GATA-1* was detected here in both the yolk sac and the allantois at E2.5, when the allantois was not yet vascularized. After E3, although *GATA-1* mRNA still was detected in the yolk sac, its expression in the allantois stopped and *GATA-3* started to be expressed there. This transcription factor was not detected in the yolk sac at the stages analyzed.

We found β major globin mRNA as early as E2.5 in the allantois, yolk sac and red blood cells. These data differ from those of Leonard *et al.* (19), who detected β major in blood only from E5.5 onward. This discrepancy may be caused by a technical difference in the RT procedure. We used oligo(dT) RT-primers to pull out cDNAs only from polyadenylated mRNAs, i.e., 1% of the total RNA. As we had very little material after retrotranscription, in some cases we amplified the transcript 25–30 times, after verifying that the amplification was still in the growing logarithmic phase, whereas Leonard *et al.*'s positive results were obtained with 21–26 cycles. In a few cases we compared cDNAs obtained with dT or random retrotranscripts. PCR amplification using random primer retrotranscripts did not uncover β major mRNA in E3 and E4 cDNA samples, in agreement with Leonard *et al.*'s results, whereas with dT cDNA it did. Furthermore we treated material from single embryos, detecting different patterns between individuals, in the case of allantoic ε and ρ mRNA expression, at the E2.5 time point but not after. The expression pattern that we found in control cell line MSB1 is identical to the pattern described by Leonard *et al.* (19).

The third finding of this work is that EC or EC precursors colonized the host bone marrow extensively after the heterospecific grafts. The contribution of allantoic grafts to host bone marrow EC in addition to hematopoietic cells and osteoclasts came as an unexpected finding. We believe that a blood-borne transport must be involved because EC homing in this system is not restricted to the vicinity of the graft, as would occur with endothelial sprouting. Indeed countercurrent migration of $QH1^+$ cells in the arteries of chicken embryos engrafted with E3-E4 quail limbs has been described (28, 29). Furthermore circulatory migration of EC or EC precursors and homing to the bone marrow recently has been demonstrated in the adult mouse (30). Our findings generate the following speculations: (*i*) blood-borne dissemination of EC precursors may be a normal developmental mechanism, different from the interstitial migration of precursors of somitic origin, which occurs earlier in ontogeny; (*ii*) EC blood-borne dissemination may be necessary for bone marrow rudiment colonization; and (*iii*) the cell that disseminates might be the

common putative precursor of EC and hematopoietic cells, the so-called hemangioblast (31, 32).

In conclusion, we present evidence that the avian allantois is, like the para-aortic region (26), a site where hematopoietic progenitors emerge from the mesoderm *de novo*. To assess the respective importance of the para-aortic region and allantois in the production of definitive HSC, an experimental scheme reproducing the normal anatomical relationships would be useful; it would be informative, if feasible, to replace the presumptive allantoic territory of a chicken embryo with that of a quail and allow the chimera to develop *in ovo*. This experimental design also might bring about information about cell migrations possibly involved in the constitution of this territory during gastrulation.

To clarify the emergence of EC, and balance the molecular approach of hematopoiesis with that of EC determination and differentiation, we are undertaking the analysis of vascular endothelial growth factor (VEGF) and VEGF-R2 expression. We also plan to determine whether HSC and EC from the allantois colonize other rudiments than the bone marrow. Finally, it remains for us to investigate to what extent these findings about the allantois may concern mammals, according to the suggestions of Downs and coworkers (9, 13) even though the allantois is held to be purely mesodermal in murine embryos (33, 34).

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