On the origin of haemopoietic stem cells in the avian embryo: an experimental approach

Par FRANÇOISE DIETERLEN-LIEVRE
Institut d’Embryologie expérimentale du Collège de France et du C.N.R.S.

SUMMARY

It is currently accepted that stem cells of the definitive blood cell lines originate from the yolk-sac blood islands. Experiments were devised to examine the validity of this theory in the avian embryo. These involved grafting two-day-old quail embryos on to chick yolk-sacs of comparable developmental stages, i.e. before or shortly after the establishment of vascularization. The conclusions of the experiments are based on the possibility of distinguishing chick cell nuclei from those of the quail.

In the developing haemopoietic organs (spleen and thymus) of quail embryos grafted on to the chick and subsequently incubated for 6–11 days, all cells, whether belonging to the granulopoietic, erythropoietic or lymphopoietic series, are of quail type. Thus these organs have not been colonized by chick stem cells.

On the other hand, coelomic graft experiments show that the development of these organs is indeed dependent on an extrinsic colonization by haemopoietic cells; quail spleen or thymus rudiment, developing in the coelom of a chick, is populated by chick cells. Thus no incompatibility which would prevent heterospecific colonization exists in this system.

It is concluded that haemopoietic stem cells of the definitive blood cell series originate from some source other than the yolk-sac, and that this source must be intra-embryonic.

INTRODUCTION

The haemopoietic organs of the embryo are populated by extrinsic stem cells (Moore & Owen, 1965). However, the origin of these stem cells is not firmly established. According to Moore & Owen (1967a; see also Metcalf & Moore, 1971), the yolk-sac blood islands are the source not only of primitive erythroblasts, but also of stem cells for definitive haemopoiesis. Though currently accepted, this theory is debated (Marks & Rifkind, 1972a, b; Harrison & Russell, 1972) and none of the experimental results brings unequivocal proof.

The strongest arguments of Moore & Owen are based on experimental work with the avian embryo (1965, 1967b). In order to trace the origin of cells, these authors use the possibility of recognizing the male chromosomal complement of mitotic cells from that of the female and search for haemopoietic cells from the partner in parabiosed embryos or in irradiated embryos injected with cell suspensions. Thus a cell suspension from 7-day yolk-sac is able to repopulate
Fig. 1. Schematic drawing of blastoderms at extreme grafting stages. The seam between the quail and the chick blastoderms is made at the dotted line, after removing the chick embryo and replacing it by the quail embryo. (A) HH. Stage 9: 7 somites (around 33 h). Blood islands are present in the extra-embryonic area, but the circulation is not closed. (B) HH. Stage 15: 24–27 somites (around 55 h). Cross-circulation is established between the embryo and the area vasculosa.

the spleen and marrow of irradiated 13-day-old embryos. However, vascularization is established prior to any of these experiments and consequently cellular traffic between the various haemopoietic primordia may already have occurred.

It therefore seemed useful to devise an approach involving experimental interference at very early stages. For this purpose, the cell recognition system of quail-chick combinations (Le Douarin, 1969) has been adopted. The experiment consists of grafting 2-day-old quail embryos on to chick blastoderms in ovo (Martin, 1972), in such a way that the grafted quail embryo develops on the chick area vasculosa. The extra- or intra-embryonic origin of the stem cells can then be determined by verifying whether the differentiating blood cell nuclei in the haemopoietic organs are chick or quail. Granulopoiesis and erythropoiesis in the spleen, and lymphopoiesis in the thymus of the experimental animals have been studied.

Two points are critical: first, the species to which the different types of haemopoietic cells belong must be identified; secondly, it must be demonstrated that haemopoietic stem cells are able to settle and differentiate in xenogeneic
Table 1. Distribution of quail embryos grafted on chick blastoderms showing grafting stage and total age reached

The figures indicate the number of experimental embryos obtained.

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organ rudiments. Le Douarin & Jotereau (1973a, b) have shown that chick and quail lymphocytes are distinguishable, and that the isolated thymic endoderm of the quail can attract chick haemopoietic stem cells and promote their differentiation along the lymphopoietic pathway. In the first two sections of the present work, it will be demonstrated that early stages of erythropoietic and granulopoietic cell lines of chick and quail are distinguishable, and that chick stem cells are able to populate the quail splenic mesenchymal primordium.

MATERIALS AND METHODS

White Leghorn chick and quail (Coturnix coturnix japonica) embryos were used. Young stages were timed according to Hamburger & Hamilton (1951). The same markers of development may be recognized in the quail and in the chick, so that the same stage nomenclature was used, even though the incubation times necessary to reach identical stages are slightly different. To study the immigration of stem cells into the spleen, the primordia of both spleen and pancreas were removed from quail embryos incubated for 3–4 days and were grafted together in the coelom of 3½-day-old chick embryos by the method of Wolff (1946). They were removed and fixed when the total age of the grafts was 11 days.

For the grafting of whole quail embryos on chick blastoderms, embryos aged 40–60 h were used, the stages of quail embryo and chick blastoderm to be associated being matched. The operation was performed at a variety of stages ranging from Hamburger and Hamilton (HH) stage 9 (5 somites) to HH stage 15–16 (27 somites) (Fig. 1 and Table 1). Complete circuits between embryonic body and area vasculosa are not established until stage 12, and until this time
there is no movement of blood cells, though the heart begins to contract at stage 10 (Hamilton, 1952). The grafting technique has been described by Martin (1972): after tearing open the vitelline membrane, the chick embryo is removed and replaced by the quail embryo which is then seamed all around by cutting together the edges of both blastoderms with paschew scissors. In order to carry over as few quail extra-embryonic blood islands as possible, the seam is made close to the grafted quail embryonic body. Eighty-six embryos were grafted, 17 of which survived. Their total age at the end of the experiment ranged from 8 to 13 days (Table 1).

Grafted spleens of the first series (coelomic grafts), whole grafted embryos or their spleen and thymuses (depending on the age) were fixed in Carnoy’s fluid. Five μm sections were stained by the Feulgen–Rossenbeck technique for demonstrating DNA, or by the Pappenheim–Unna technique to demonstrate nuclear and cytoplasmic basophilia. Bone marrow and bursa of Fabricius were not studied, since they differentiate at later stages and are still very immature at 13 days.

RESULTS

I. Identification of quail and chick haemopoietic cells

Le Douarin (1969, 1973) has shown that quail cell nuclei possess one or more prominent DNA-containing nucleoli. This makes it possible to identify individual cells in Feulgen–Rossenbeck-stained sections after experimental intermixture of quail and chick cells. Quail and chick lymphocytes, the only blood cells yet studied from this point of view (Le Douarin & Jotereau, 1973a, b) are readily distinguished; the former have a single thick, irregular heterochromatic mass plus some finer DNA material around the margin of the nucleus, whilst the chick lymphocyte nucleus has numerous, finely dispersed chromocentres.

Nuclear markers of the granulopoietic and erythropoietic cells have been studied in the spleen, where, during embryonic life, erythropoiesis proceeds in the sinusoids, granulopoiesis in the mesenchymal reticulum (DeLanney, Ebert,
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Coffman & Mun, 1962). Lymphocytes are not present until after hatching. Up to day 13, the latest stage studied here, the spleen in both species remains relatively embryonic. White and red pulp have not begun differentiating, and granulocytes or granuloblasts still occur in small numbers. In the chick, the splenic tissue is a loosely meshed mesenchymal reticulum with large sinusoids; in the quail it is dense with small sinusoids (Figs. 2–5). The mesenchymal reticulum is essentially composed of undifferentiated cells which appear homogeneous when stained by the Feulgen–Rossenbeck technique. In the chick they have round or elongate nuclei with two to six very fine chromocentres. In the quail, mesenchymal cell nuclei are also round or elongate; round nuclei have one thick central heterochromatin mass; elongate ones have three or four.

Many of these mesenchymal cells are precursors of the granulocytes and since these are relatively rare at the stages studied, their species identification will not be discussed here. In methyl green-pyronin-stained sections, one class of cells with a characteristically heavy cytoplasmic pyroninophilia appears prominently among the undifferentiated ones. These cells are termed haemocytoblasts (Dantschakoff, 1916; DeLanney et al. 1962) or stem cells (Metcalf & Moore, 1971). They are located either in the sinusoids or in the mesenchyme. In the chick they are round and large; their massive nucleus has a prominent pyroninophilic nucleolus. In Feulgen-stained sections, a small chromocentre is seen in association with the bulky nucleolus, whilst several very small DNA masses are situated at the periphery of the nucleus (Fig. 2). In the quail these cells are rare; both the cytoplasmic and nuclear outlines are irregular, and their nucleus contains a large DNA mass, which is often linked to the nuclear membrane (Fig. 3).

As differentiation of red cells proceeds, their chromatin becomes increasingly condensed; it is for this reason that erythropoietic cells are readily identifiable only in their early stages. Chick erythroblast nuclei have a number of rather homogeneous, evenly distributed chromatin lumps (Fig. 4); those from quail have one large central DNA mass and a peripheral ring of condensations encircling the whole nucleus (Fig. 5).

In summary, at some stages of their evolution, differential nuclear features

Figures 6–8
Quail spleen rudiments developed up to 12 days in the chick coelomic cavity, after transplantation at different stages.

Fig. 6. Transplantation at HH stage 22 (4 days). Most nuclei contain the characteristic quail nucleolus.

Fig. 7. Transplantation at HH stage 20. Quail nuclei are present only in small areas (circled) and chick nuclei in the others.

Fig. 8. Transplantation at HH stage 19. Another example of mosaic tissue, composed of chick and quail cells. Typical chick erythropoiesis is under way in the sinusoid. Chick haemocytoblasts are present in the mesenchyme and in the sinusoids (arrows). Quail cells are circled.
Avian embryo blood stem cells make it possible to discriminate between chick and quail cells of the lymphopoietic, granulopoietic and erythrophoietic series. In addition, all cell types are smaller, both in nucleus and cytoplasm, in the quail than in the chick.

II. Pattern of differentiation of the spleen rudiment as a xenogeneic intracoelomic graft

The aim of this section is to demonstrate that the quail spleen rudiment may be colonized by chick haemopoietic cells, and that indeed, depending on the timing of the experiments, such a colonization is essential for the development of the organ. During the grafting period, the spleen separates from the pancreas with which it was continuous at the time of explantation and assumes its normal round or ovoid shape. The younger the transplant the richer it appears microscopically in chick cells at the end of the experiment (Figs. 6 and 7). Many chick cells can be classified as haemocytoblasts, due to their highly pyroninophilic cytoplasm and their prominent pyroninophilic nucleolus. This nucleolus shows only a small DNA component, thus identifying the cell as a chick cell. The haemocytoblasts are seen both in the sinusoids and in the mesenchymal reticulum of the spleen (Fig. 8). In the reticulum, the chick cells are grouped together into large areas, whilst quail cells predominate in some other zones. Granulocytes are present in some regions. The organs developed from the younger transplants (HH stage 19) show very few quail cells, which are usually located along the sinusoids. The quail pancreas, grafted simultaneously with the spleen, is never invaded by chick cells. The same remark applies to a number of other quail tissues (personal observation).

III. Pattern of differentiation of the spleen and thymus in quail embryos grafted on to chick blastoderms

Table 1 provides the grafting stages and total ages reached by the experimental embryos. A thorough examination of the haemopoietic organs reveals identical results in all embryos but one (Figs. 9-12).

The general appearance of the spleen is typical of the quail. In younger embryos, the splenic tissue is a homogeneous, dense, mesenchymal network, with narrow sinusoids; pyroninophilic haemocytoblasts may occasionally be seen in the sinusoids. The cells of the mesenchymal network all look alike and possess in their nucleus a Feulgen-positive, thick central nucleolus typical of the quail. In older embryos, the degree of development varies. In more advanced

Figures 9–11

Fig. 9. Thymus of a 13-day-old quail embryo developed on a chick area vasculosa. Lymphocyte nuclei are all of quail type.

Fig. 10. Spleen of a 13-day-old quail embryo developed on a chick area vasculosa. All cells are of quail type.

Fig. 11. Idem. Erythropoiesis (arrows) is also of quail type.
ones, differentiation of sheaths around arterioles is beginning. Haemocyto-
blasts are more frequent than in younger embryos and the splenic parenchyma
bordering the sinusoids appears diffusely pyroninophilic. All identifiable cell
types, after Feulgen-staining, are seen to belong unmistakably to the quail
species, whether they are incorporated in the mesenchymal reticulum, or whether
they are early erythropoietic cells (Figs. 10 and 11). In the thymus of these
embryos, all cells, i.e. lymphocytes, reticular cells and connective cells, have
quail nuclei (Fig. 9).

However in one embryo – out of 17 – grafted at HH stage 15 and maintained
until 12½ days of incubation, the spleen contains two small groups of chick-type
cells (Fig. 12). One of these groups is made up of six to eight cells, the other,
near the first, of two to three cells. The thymus of this embryo is entirely
composed of quail cells.

Thus, in 16 out of 17 embryos, the haemopoietic organs of quails developing
on chick blastoderms contain only quail cells. It thus follows that no stem cells
originating from the chick extra-embryonic area vasculosa have settled in the
rudiments of these organs. Since the quail area vasculosa is absent by virtue of
the experimental design, quail stem cells must have been available from some
source other than the area vasculosa.
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One exception concerns an embryo grafted after establishment of the circulation between the area vasculosa and the chick embryo: in this case traffic of haemopoietic cells may well have occurred before the grafting of the foreign embryo.

DISCUSSION

The experiments reported in this work have been devised in order to investigate the theory of the yolk-sac origin of definitive blood stem cells in the avian embryo. The timing of colonization of the quail spleen implanted in the chick coelom will be discussed first.

Moore & Owen (1965) have demonstrated that there is an extensive traffic of haemopoietic cells within the chick embryo and that haemopoietic organs are 'largely populated by blood-borne cells'. The coelomic graft experiments described here show that 3- or 4-day quail spleen rudiments are colonized by chick cells. The chick cells either settle in the sinusoids, where they undergo erythropoiesis, or enter the splenic mesenchyme, where some of them will later differentiate into granulocytes. In grafts developed from the younger rudiments, foreign chick cells are so numerous that quail cells appear scattered; thus it is likely that all haemopoietic elements have an extrinsic origin and that colonization, which either has not, or has only just begun on day 3, is already well under way at day 4. This event happens earlier in the spleen than in the thymus, which in the quail, is colonized on days 6 and 7 of incubation (Le Douarin & Jotereau, 1973a, b). Some chick cells have the appearance of undifferentiated splenic mesenchymal cells, indicating that extrinsic haemopoietic cells, upon entering the splenic mesenchyme, lose (at least temporarily) distinctive features of blood cells. Two points should be emphasized. First, entry of haemocytoblasts into the grafts is restricted to haemopoietic tissue; in other words haemopoietic tissue selectively attracts and/or retains these cells: differentiation of the attracted haemocytoblasts is directed along pathways normally specified by the grafted tissue, i.e. lymphopoiesis in the thymus (Le Douarin & Jotereau, 1973a, b), erythropoiesis and granulopoiesis in the spleen. Secondly, any immune reactions, which might occur, inhibit neither attraction of chick haemopoietic cells by quail haemopoietic organ rudiments nor their subsequent differentiation during the experimentation period.

Therefore, absence of chick cells from the thymus and spleen of quails grafted on chick yolk-sacs has only one obvious explanation: i.e. stem cells for the definitive blood cell series do not originate from the yolk-sac blood islands and they must arise within the embryo proper. Thereafter they enter the blood traffic and may be found in the yolk-sac vessels. Against this interpretation, one could object that the grafted embryo has carried with it a fragment of its own yolk-sac, or may have regenerated some. However, even assuming this to be the case, the relative amounts of chick and quail yolk-sacs would be overwhelmingly in favour of the former. Competition between quail and chick stem cells, favourable to isogeneic cells, might further be invoked. The presence of two
small groups of chick cells in one exceptional quail embryo answers that argument; this embryo was grafted at a late stage, approximately 10 h after vascularization had been established. Stem cells which had differentiated in the chick embryo had probably travelled to the yolk-sac and were present there when the quail embryo was grafted; these chick cells migrated to the quail embryo and were trapped in the spleen. In fact it seems likely that each of the two small groups of chick cells is the progeny of a single stem cell. This exceptional case shows that, in this particular experimental situation, heterospecific colonization is possible. The fact that heterospecific colonization does not happen after early grafting, must mean that no stem cells for the definitive haemopoietic series are present at that time in the yolk-sac, and that none is produced during subsequent development. Thus the yolk-sac blood islands’ production must be limited to primitive erythroblasts.

Another question arises: why, out of four embryos grafted at stage 15 and one at stage 16, did only one show heterospecific colonization, circulation being closed around stage 12? Two possibilities may be considered: either definitive stem cells only begin differentiating around stage 15, or they do not immediately enter the blood stream. Individual variations in parameters of differentiation being the rule in outbred chick embryos, it is not too surprising that one embryo may be in advance as regards presence of stem cells in its circulation. Whatever the reason, it is most probable that, at later stages, wandering definitive stem cells become more numerous in the circulation and consequently in the yolk-sac. Moore & Owen (1967b) used 7-day yolk-sac cell suspensions to reconstitute the haemopoietic organs of irradiated chick embryos, but there is no evidence that stem cells present in these suspensions originally differentiated in the yolk-sac.

In conclusion, the experimental results reported here exclude a yolk-sac origin of definitive blood stem cells in the avian embryo. Hollyfield (1966) reached a similar conclusion in amphibians. The site of origin of definitive stem cells remains hypothetical. They probably differentiate within the extensive erythropoietic foci present in the general mesenchyme of the embryo. These foci, first described long ago (Dantschakoff, 1908; Sabin, 1920), extend all around the dorsal aorta of the chick, from neck to tail, and are especially abundant in the vicinity of the lung and spleen. Experiments are under way to test their capacity as definitive stem cell suppliers.

RESUME

D’après la théorie de Moore & Owen (1967), les cellules souches des lignées sanguines définitives seraient issues des îlots sanguins du sac vitellin embryonnaire. Cette théorie est mise à l’épreuve chez l’embryon d’Oiseau grâce à la possibilité de distinguer les cellules de Poulet des cellules de Caille. Des embryons de caille de 2 jours d’incubation sont greffés sur l’aire vasculaire d’embryons de poulet de stade correspondant, c’est à dire peu avant ou peu après l’établissement de la circulation.

Dans les organes hématopoïétiques (rate et thymus) de la caille greffée, incubée jusqu’à 13 jours, toutes les cellules sont de type caille, qu’il s’agisse de cellules granulopoïétiques,
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erthropoïétiques ou lymphopoïétiques. Ces organes n'ont donc pas été colonisés par des cellules souches sanguines de Poulet, qui proviendraient des îlots sanguins extra-embryonnaires.

Par ailleurs, des expériences complémentaires montrent que le développement de ces organes dépend bien d'une colonisation par des cellules hématopoïétiques extrinsèques et que la colonisation hétérospezificique n'est pas impossible: en effet l'ébauche splénique de caille, transplantée en greffe coelomique chez le poulet, se peuple de cellules de poulet; il en est de même pour le thymus.'

L'ensemble de ces résultats permet de conclure que les cellules souches hématopoïétiques des lignées sanguines définitives ont une autre origine que le sac vitellin, et que cette origine doit être intra-embryonnaire.

REFERENCES


(Received 1 July 1974)