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# Origin of hemopoietic stem cells in embryonic bursa of Fabricius and bone marrow studied through interspecific chimeras

(embryonic chimeras/differentiation/histogenesis)

N. M. LE DOUARIN, E. HOUSSAINT, F. V. JOTEREAU, AND M. BELO

Laboratoire d'Embryologie, Université de Nantes, 38, Bld Michelet B.P. 1044–44037, Nantes Cedex, France

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**ABSTRACT** **The histogenesis of the bursa of Fabricius and of bone marrow was studied by a biological cell marking technique based on differences in the nuclear structure of two species of birds, Japanese quail *(Coturnix coturnix japonica)* and chick *(Gallus gallus)*. In quail cells the nucleus contains a large amount of heterochromatin associated with the nucleolus. That makes it possible to distinguish them from chick cells after Feulgen–Rossenbeck staining and by electron microscopy. By grafting bursal rudiments and limb buds of quail into chick and inversely it was possible to demonstrate that the whole hemopoietic population of the bursa of Fabricius and of bone marrow is derived from blood-borne extrinsic stem cells. Neither endoderm nor mesoderm of the bursal rudiments is capable of differentiating into lymphoid cells. Combinations of quail bursal endoderm with chick homologous mesenchyme showed that the reticular cells of the follicles are the only endodermal derivatives of the bursa. The mesenchymal bursal component gives rise to the interfollicular connective cells.**

**The contribution to bone marrow histogenesis of cells of vascular and blood origin, on one hand, and of the elements of the cartilaginous model, on the other hand, was analyzed. It appeared that osteoblasts, osteocytes, and stromal cells of marrow are derived from the perichondrium. In contrast, the endothelium of the vascular buds and the hemopoietic cells which invade the diaphysal cartilage during the endochondral ossification process do not belong to the mesenchymal bone primordium but have a fully extrinsic origin.**

Hemopoietic stem cells can be defined as elements that give rise to the specialized cells of blood that, in addition, have a capacity for extensive proliferation resulting in renewal of their own kind. The question then arises of whether the stem cell, although committed to hemopoiesis, is able to give rise to various strains of blood cells or whether its developmental capabilities are to a certain extent restricted to particular differentiating pathways. If the embryonic hemopoietic stem cell were multipotential, it must be assumed that its differentiation into lymphoid and myeloid cell lines is induced by the microenvironment provided by each blood-forming site.

In both avian and mammalian embryos, the first hemopoietic cells are formed in the early yolk sac. In mammals, hemopoiesis in the yolk sac is succeeded by blood cell formation in the liver and spleen, and finally, in both birds and mammals, hemopoiesis is established in bone marrow, where it persists throughout life. It has long been considered that this progression of the hemopoietic sites results from *in situ* differentiation of new stem cells proper to each intraembryonic blood-forming organ (1, 2). Blood cells were supposed to derive either from endodermal cells, in the thymus (3–6) and in the bursa of Fabricius (7), or from mesenchymal or vascular cells (8–11). Finally, several authors have even attributed a parenchymal origin to liver fetal hemopoiesis (12, 13).

However, an initial observation of Owen (14) showed that placental interchange of blood in twin cattle results in blood chimerism in adult life. This seemed to indicate that for a period, stem cells coming from one or several primary hemopoietic sites are present in the blood stream of the embryo and settle down in blood-forming tissues. Experimental support for the concept of stem cell migration was provided by the use of chromosomal markers to demonstrate the passage of stem cells across the circulatory connection between parabiosed chick embryos (15). After the onset of vascular anastomosis between a male and a female chick embryo, sex chromosomal analysis showed a chimerism in dividing thymic (16) and bursic lymphocytes (17). Similar observations were reported later for bone marrow by Metcalf and Moore (18).

A cell marking technique devised by one of us (19, 20) made it possible to study the embryological origin of the various thymic cell types. It was shown that thymic endoderm, which is determined early, has a strong attractive capacity for blood-borne hemopoietic stem cells which are trapped in the developing endodermal reticulum of epithelial cells even if the endoderm is grafted to a heterotopic location. Moreover, it was demonstrated that the whole lymphoid thymic population of embryonic and early postnatal stages depends on extrinsic seeding stem cells that invade the endomesodermal anlage in two successive inflows: the first during thymic histogenesis and a second which, around hatching time, completely renews the first lymphoid cell population (21; *).

The cell marking technique we use is based on structural differences between the interphase nucleus in two species of birds closely related in taxonomy, Japanese quail *(Coturnix coturnix japonica)* and chick *(Gallus gallus)*. In quail cells the nucleus contains one or several large heterochromatic condensations associated with the nucleolus, while in the chick the chromatin is evenly distributed in the nucleoplasm during the interphase and the amount of nucleolus-associated DNA is small. Because of these differences it is easy to distinguish quail from chick cells in chimeric embryos of the two species, especially after Feulgen–Rossenbeck staining (Fig. 1) or by electron microscopy. It seemed interesting to apply the "quail–chick" marker system to investigate the

Abbreviations: F.R. stain, Feulgen–Rossenbeck stain; CAM, chorioallantoic membrane.

* N. Le Douarin and F. Jotereau (1975), submitted for publication.

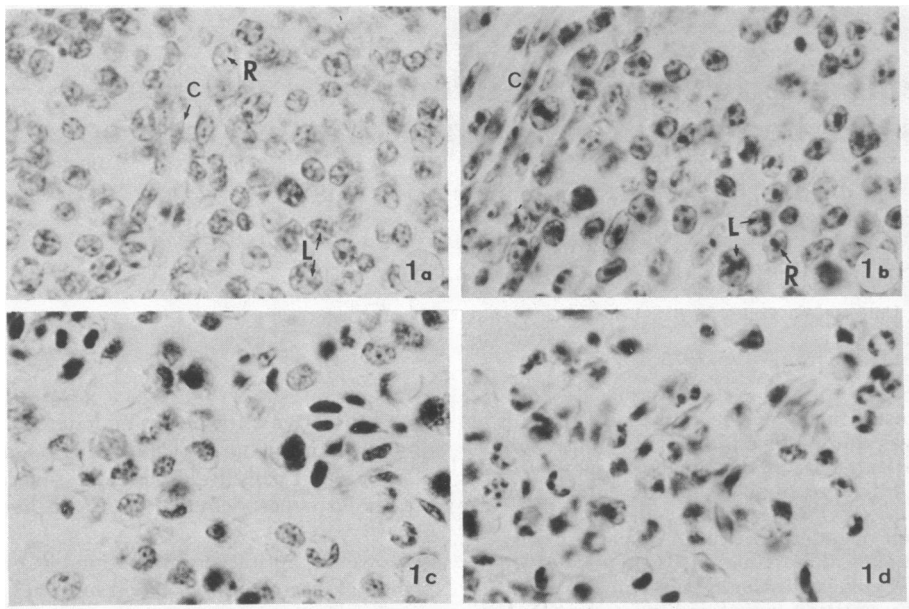


FIG. 1. Feature of chick and quail cells in bursa of Fabricius and bone marrow after Feulgen–Rossenbeck (F.R.) staining; ×845. (a) 18-day chick embryo bursa. The lymphocyte (L) nuclei show several small chromocenters. Reticular cells (R) have a clear nucleus. C = Interfollicular connective cells with elongated nuclei. (b) 16-day quail bursa. The lymphocyte (L) nuclei exhibit a very large and irregular central DNA mass and often several smaller ones attached to the nuclear membrane. Reticular cell nuclei (R) show one central, usually spheric, mass of heterochromatin. (c) 16-day chick embryo femoral bone marrow. Extravascular granulocytopoiesis. Nuclei with a network of chromatin showing small dispersed chromocenters. (d) 15-day quail embryo femoral bone marrow. Heterochromatic DNA patches are present in the nucleus at all stages of granulopoiesis.

contribution of "*in situ*" as opposed to immigrant, cells to the process of bursa and bone marrow differentiation.

The experimental procedure involved interspecific grafts of bursal and bone rudiments between quail and chick embryos at various developmental stages and the subsequent analysis of the differentiated organs to ascertain the presence of various cell types from the two species. It was shown that the whole bursa and bone marrow hemopoietic population is derived from immigrant stem cells. Analysis of different types of chimeric bursas and bones made it possible to follow the developmental fate of the various cell types forming the undifferentiated embryonic rudiments.

## MATERIALS AND METHODS

**Bursa of Fabricius.** Epithelio-mesenchymal rudiments were taken from 5- to 11-day quail embryos and grafted into 3-day-old chick somatopleure (Fig. 2). At the time of fixation the total age (age of grafting time + duration of the graft) of the bursa was 18 or 19 days. Reverse grafts of 7- to 9-day chick bursas into quail were carried out under the same conditions. The grafted tissue was fixed in Zenker's fluid and stained according to the Feulgen–Rossenbeck's procedure (22).

In a second experimental series, bursas were taken during the seventh day of incubation in chick embryos and grafted for 5 and 6 days into the somatopleure of a 3-day quail. The panoptic technique (23) was applied to the grafts in order to detect the basophilic cells previously described by several authors in the bursal rudiment (8, 18). Subsequently, the sections first observed and photographed were then treated for DNA staining. That made it possible to determine whether the basophilic cells were of quail or of chick origin.

In a third experiment, the endodermal and mesenchymal components of 7-day chick and quail bursas were separated by trypsinization. Then, quail bursal endoderm was recombined with chick mesenchyme. The two rudiments were cultivated on a semisolid agar medium for 12 hr and were grafted on the chorioallantoic membrane (CAM) of 5-day chick embryos for 14 days.

**Bone Marrow.** Limb buds taken from 4-day-old chick [stages 23–25 of Hamburger and Hamilton (24)] and 4-day-

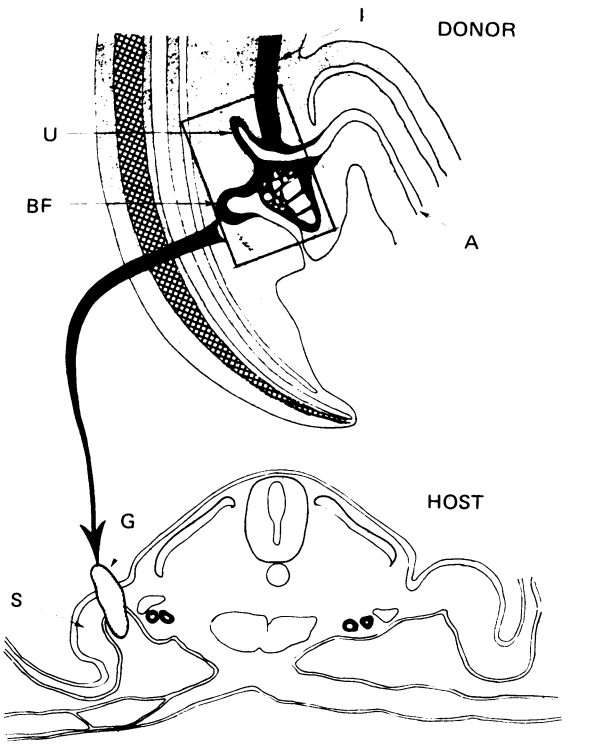


FIG. 2. Schema showing the transplantation of the endomesodermal bursal rudiment of a 6-day chick embryo into the somatopleure (S) of a 3-day host. The whole cloacal region is transplanted. U, ureter; I, intestine; A, allantois; BF, bursal rudiment; G, graft.

Table 1. Results of the graft of quail bursal rudiments from 5- to 11-day-old embryos into 3-day chick hosts

| Stage of development (days) | Duration of graft (days) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| 5 | | | | | | | * | * |
| 6 | | | | | | * | * | |
| 7 | | | | | † | † | | |
| 8 | | | | † | † | | | |
| 9 | | | † | † | | | | |
| 10 | | ‡ | ‡ | | | | | |
| 11 | ‡ | ‡ | | | | | | |

The total age of the bursa at the time of fixation is 18–19 days. Lymphoid cells are exclusively of host type when the bursa is taken at 5 and 6 days of incubation. Lymphocytes are a mixture of host and donor cells when the graft is done at 7–9 days; and solely of donor type when the bursa is transplanted from 10 days onwards.

* Host.

† Host + donor.

‡ Donor.

old quail [stages 18–19 of Zacchei (25)] were respectively grafted into the CAM of 5-day quail and chick embryos for 10–12 days. The limb was fixed in Zenker's fluid and cut into 5 $\mu$m serial sections stained for DNA.

## RESULTS

### Bursa of Fabricius

**Differentiation of Bursas of Various Ages in a Heterospecific Host Embryo.** The results of the first experimental series, which concerned the graft of quail bursas at various developmental stages into chick, are reported in Table 1. It appears that the species specificity of the grafted bursal lymphoid population depends on the stage at which the organ has been transferred from donor to host embryo. If the bursa is taken from the quail before the end of the seventh day of incubation, its lymphocytes are entirely of host origin (Fig. 3). When the bursa is isolated between the seventh and the eleventh day and then grafted into a chick, it contains a mixture of host and donor lymphoid cells. The older the bursa is at the time of the graft, the fewer the number of host cells in the follicles. Considering equally developed bursas at grafting time, the longer the transplantation, the more numerous are the host lymphocytes. The chimerism observed is different from one follicle to another. In the various experimental series, we found follicles where lymphocytes were mostly of one kind (host or donor) while, in the same organ, other follicles showed a mixture of cells belonging to the two species. The proportion of follicles with host, host + donor, or donor lymphocytes was evaluated in quail bursas grafted into chick between 7 and 10 days. The results (Table 2) show that the proportion of follicles containing host lymphocytes increases, whereas the number of follicles with only donor lymphoid cells decreases with the age of donor embryo. It is interesting to notice that in normal development, follicle formation does not occur at the same time in the whole bursal rudiment, and that can account for the heterogeneity observed in lymphoid population of the follicles in grafted bursas. A decreasing gradient of differentiation takes place from the apex to the base of the organ during the morphogenetic process. It can be assumed that the epithelium has to reach a certain degree of maturation to be able to attract the basophilic cells which are in the mesenchymal bursal component. If the bursa is grafted when the donor age is 11 and 12 days, it contains nearly exclusively quail lymphocytes (Fig. 4).

In the reverse graft of chick bursas into quail, lymphocytes were of host type when the transfer occurred at 7 days of donor age. When the bursa was taken from 8- and 9-day-old chicks, lymphoid cells of the grafted tissue were a mixture derived from both host and donor embryos.

These results indicate that the endomesodermal cells of

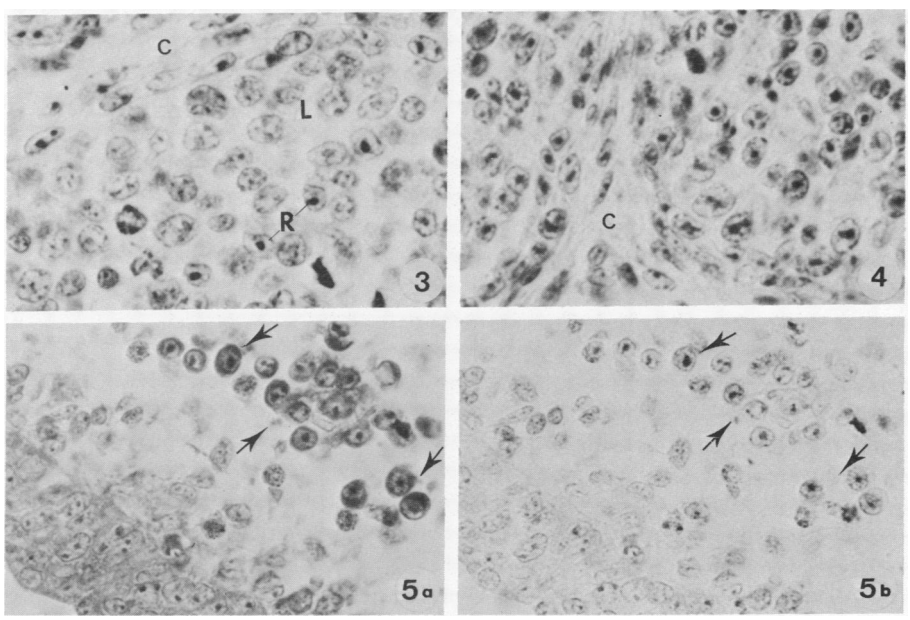


FIG. 3. Five-day quail bursal rudiment grafted for 14 days into a chick embryo. Connective interfollicular cells (C) and follicular reticular cells (R) belong to the quail species, whereas lymphocytes (L) belong to the chick host. F.R. staining; ×910.

FIG. 4. Eleven-day quail bursa grafted into a chick for 8 days. Connective (C), reticular, and lymphoid cells are of donor type. F.R. staining; ×845.

FIG. 5. Seven-day chick bursa grafted for 6 days into a 3-day quail embryo. Basophilic cells stained with the panoptic technique (a) are in the mesenchyme and near the epithelium basement membrane. Post-staining of the same section according to the Feulgen–Rossenbeck procedure shows that the basophilic cells belong to the host, since they show the quail nuclear marker *(arrows)* (b). F.R. staining; ×487.5.

Table 2. Percentage of chimeric follicles in quail bursas grafted into chick at various developmental stages

| Stage of development of donor embryo (days) | Lymphocyte chimerism (%) in bursal follicles | | |
|---|---|---|---|
| | Host lymphocytes | Host + donor lymphocytes | Donor lymphocytes |
| 7 | 0 | 44 | 56 |
| 8 | 26 | 54 | 20 |
| 9 | 36 | 53 | 11 |
| 10 | 90 | 10 | 0 |

Total age of the bursas at the time of fixation, 18 days.
* Calculated from the observation of three grafted bursas. Ninety follicles are counted for each case.

Table 3. Percentage of basophilic quail cells in mesenchymal and endodermal components of bursas taken from 7-day chick embryos and grafted into 3-day quails for 5, 6, and 7 days

| Localization of quail basophilic cells | Duration of graft (days) | | |
|---|---|---|---|
| | 5 | 6 | 7 |
| In bursal mesenchyme (%) | 91.9 | 89.3 | 65.1 |
| In contact with epithelial basement membrane (%) | 5.9 | 5.2 | 14.5 |
| Inside the endoderm (%) | 2.2 | 5.5 | 20.4 |
| Total no. of cells counted | 321 | 503 | 241 |

The enumeration was done on 10 sections per bursa, and 2 grafts were observed in each experiment.

the cloacal bursal primordium do not have the potentiality to differentiate into lymphocytes. Consequently, lymphoid differentiation of the bursa of Fabricius entirely depends on "homing" of hemopoietic cells that originate from an extrinsic stem cell source. The results provided by heterospecific grafts demonstrate that the stem cell inflow begins at the end of the seventh day of incubation and persists until the eleventh day in the quail. Later on, few stem cells seem to enter into the bursa, the main process being, from this stage, lymphocyte proliferation and differentiation.

**Short-Time Graft Experiment.** In the experimental series of short-time grafts of chick bursas into quail, basophilic cells showing the quail nuclear marker were found in both mesenchyme and endoderm (Fig. 5). The respective distribution of basophilic cells in the two bursal components in the 12- to 14-day bursas (7 days at the time of the graft + 5, 6, or 7 days in the host) are indicated in Table 3. The results show that numerous basophilic cells remain in the mesenchyme a long time after the beginning of stem cell inflow, and that the invasion of endoderm takes place slowly. In the grafted bursas, abundant granulopoiesis was observed, as in normal organs of the same age. Granulopoietic cells show the quail nuclear marker and, just as do the lymphocytes, they are derived from immigrant basophilic stem cells.

**Heterospecific Association of Endodermal and Mesodermal Bursal Rudiments.** Quail bursal endoderm and chick bursal mesenchyme from 7-day-old embryonic rudiments were associated in culture (12 hr *in vitro* + 14 days on CAM). Thirty-five grafts were done into 3-day chick embryo hosts. Twenty-five explants could be found at the completion of the experiment and observed histologically. In 22 cases normal bursal tissue developed and were composed as follows: endoderm lining the internal bursal lumen and reticular cells of the follicles exhibited the quail nuclear marker, whereas the lymphoid population and interfollicular mesenchymal cells were derived from the chick host (Fig. 6).

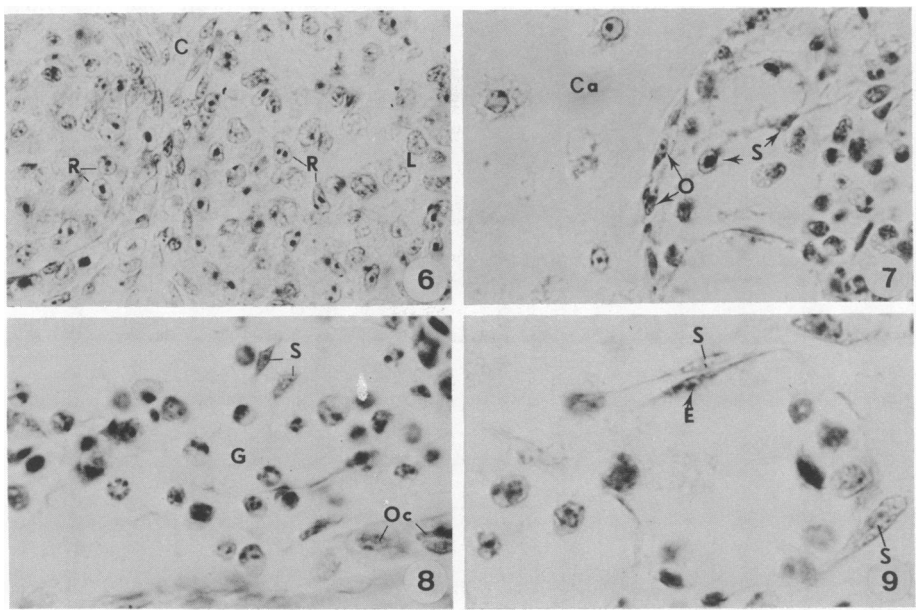


FIG. 6. Heterospecific combination of quail endoderm with chick mesenchyme of 7-day bursal rudiment. Culture 12 hr *in vitro* + 14 days on chick CAM. Connective (C) and lymphoid (L) cells belong to chick species. Epithelial reticular cells of the follicles (R) show the quail marker. F.R. staining; ×650.

FIG. 7. Femoral bone-marrow differentiated in a 4-day quail limb bud grafted into the somatopleure of a 3-day chick for 12 days. Cartilage cells (Ca), osteoblasts (O), and stroma cells (S) are of quail type. Hemopoietic cells are of host type. F.R. staining; ×780.

FIG. 8. Femoral bone marrow differentiated in a 4-day chick limb bud grafted into the somatopleure of a 3-day chick for 12 days. Osteocyte (Oc) and stroma cells (S) are of chick type. Hemopoietic cells (G) belong to quail. F.R. staining; ×650.

FIG. 9. Same experiment as represented in Fig. 8. Stroma cells (S) belong to the donor (i.e., chick); endothelial cells (E) of the vascular bud show the quail nuclear marker. F.R. staining; ×845.

### Bone marrow

Heterospecific grafts of limb buds between quail and chick embryos result in the development of long bones, which were observed after Feulgen–Rossenbeck staining. In both combinations (quail limb grafted into chick or inversely), cartilage cells, osteoblasts, and osteocytes were of donor origin (Fig. 7). Endothelial cells of the vascular buds invading the cartilaginous bone rudiments were of host type; such was also the case for all hemopoietic cell populations of the marrow which differentiate in the bone cavities (Figs. 8 and 9). Hemopoiesis takes place both intra- and extravascularly. Granulopoiesis essentially occurs in the network of the stroma. In the various steps of granulocyte differentiation, quail and chick cells can be recognized by their nuclei. Concerning the erythropoietic process, the various developmental stages of erythrocyte differentiation can be distinguished except the latest ones involving nuclear pycnosis, which impairs the quail cell marker. The marrow stroma made up of a loose mesenchymal tissue located between vascular buds and ossification trabeculae are built with donor cells (Figs. 8 and 9).

## CONCLUSIONS AND DISCUSSION

The experiments reported above show that hemopoietic differentiation of both bursa of Fabricius and bone marrow depends entirely on an immigration process of stem cells. Previous studies based on the use of chromosomal markers (see ref. 18 for review) had led to a similar conclusion, but the contribution to lymphopoiesis of cells belonging to bursal and marrow rudiments could not be excluded because this technique allows only for analysis of the origin of dividing cells. Using the quail–chick marker system, which makes it possible to visualize the whole cell population of the organ at a given time, we were able to demonstrate that neither mesenchyme nor endoderm of the bursal rudiment has the ability to undergo lymphoid differentiation. From the end of the sixth day of incubation in the quail and from the seventh day in the chick, bursal rudiments attract circulating stem cells, which begin to invade the mesenchyme. A certain number of hemopoietic cells stay in the bursal mesenchyme, where they differentiate into granulocytes. Some of them penetrate into the epithelium and later on multiply and differentiate into lymphocytes. The basophilic cells that appear in the bursa rudiment are actually the extrinsic blood-forming stem cells. Chick bursal anlagen grafted into quail embryos before the time of their invasion by the homospecific hemocytoblasts, indeed show basophilic cells containing the quail nuclear marker and are subsequently populated only by quail granulocytes and lymphocytes. The same observation has been reported for the thymus (*; 26). The seeding of stem cells in the bursal rudiment proceeds as an extensive inflow that starts in the quail and in the chick 24 hr after the beginning of thymic colonization, the latter occurring from 5 to 6 days in the quail (during the sixth day of incubation) and from 6.5 to 8 days in the chick (during the second half of the seventh day and the full eighth day of incubation). The invasion process lasts longer for the bursa than for the thymus; the stem cell inflow becomes significantly lower in the quail only after the eleventh day of incubation.

Through heterospecific associations of quail bursal endoderm with chick homologous mesenchyme it was demonstrated directly that follicular reticular cells, which provide the environmental conditions convenient for B-lymphocyte differentiation, are derived entirely from the endodermal bursal component.

Bone marrow formation is related to the process of bone differentiation from a cartilage model. Although the mechanisms of endochondral ossification, as they appear from heterospecific grafts of limb buds between quail and chick embryos, will not be analyzed in detail in this article, certain conclusions concerning the formation of the bone marrow may be drawn. Our observations show that the endothelium of the blood vessels and the hemopoietic stem cells originate from mesenchymal cells that do not belong to the bone-to-be rudiment. On the contrary, the osteogenic cells and the perivascular mesenchyme that give rise to the so-called stroma of the bone marrow are derived from the bone mesenchymal anlage. The capillary buds invading the cartilage model bring, inside their lumens, the hemopoietic stem cells, and outside their walls they carry along mesodermal elements from the perichondrium or from the mesenchyme surrounding the cartilaginous rudiment. The latter give rise both to the osteoblasts and to the marrow reticular cells. Secondarily, blood-forming cells cross the endothelial wall and spread into the network of reticular cells, where they undergo hemopoietic differentiation.

These observations extend to the bursa of Fabricius and the bone marrow the data previously obtained concerning the thymus, i.e., the primary seeding of embryonic hemopoietic organs by blood-forming stem cells via the circulation.

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