TRACING OF CELLS OF THE AVIAN THYMUS THROUGH EMBRYONIC LIFE IN INTERSPECIFIC CHIMERAS*

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Various types of immunological deficiency diseases are related to developmental anomalies of primary lymphoid organs (1, 2). Since the thymus is directly concerned with the acquisition of cellular immunity (3), the normal processes of thymic differentiation have been investigated on many occasions. The results, however, have been very controversial concerning the embryological origin of thymic lymphocytes (4). This paper deals with an experimental analysis of the evolution of the cell components found in the thymic primordium of birds during embryonic life.

The thymus derives from an epitheliomesenchymal rudiment originating from the 3rd and 4th pharyngeal pouches (5-10). In the chick embryo, the thymic endoderm separates from the pharynx during the 5th day of incubation as a cord of epithelial cells which elongates along the jugular vein (9, 10). A thin mesenchymal capsule surrounds the endodermal primordium and the mesenchymal cells penetrate it, lobulation and vascularization of the organ occurring together. Around the 11th day of development lymphoid differentiation of the thymus becomes evident.

According to one view the lymphocytes originate by transformation of the epithelial cells of the early anlage. This idea initially formulated by Kolliker (11) was supported by several authors who claimed to demonstrate transitional forms between thymic epithelial and thymic lymphoid cells (10, 12-17). From an experimental study of the histogenetic capacities of the early mouse endodermal thymic rudiment, Auerbach (18, 19) also concluded that the epithelial component was the source of thymus lymphocytes. According to the "substitution" theory proposed by Hammar (20) and supported by many subsequent studies (9, 21, 22) thymic lymphocytes were derived exclusively from connective tissue lymphocytes which invaded the early epithelial anlage. By experiments carried out in the chick embryo, Moore and Owen (23, 24) demonstrated the existence of vascular migration pathways of chromosomally labeled cells invading the thymus in 7- to 8-day-old embryos. According to these authors the thymus receives an inflow of blood-borne stem cells, presumably originating from the blood islets of the yolk sac, which proliferate and differentiate into lymphocytes after reaching the thymus (25).

Since chromosomal markers give information only about dividing cells, it seemed interesting to apply to the problem of thymus histogenesis, a cell labeling technique which, by simple histological observations, allows for the evaluation of the composition of the whole cell population of thymic tissue at a given time. Le Douarin has recently devised a new biological cell marking technique (26-28) which can be employed in the study of embryonic cell migrations (see 29 for references). This technique is based on structural differences in the interphase nucleus of two species of birds closely related in taxonomy:

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the Japanese quail (*Coturnix coturnix japonica*) and the chick (*Gallus gallus*). In the Japanese quail a large amount of heterochromatic DNA is associated to the nucleolus so that this organelle is hypertrophied in all embryonic and adult cells of this species. This feature is not usual in birds or mammals (29) and especially not in the chick, in this species, the chromatin is evenly dispersed in the nucleoplasm with some small chromocenters and participates only little to nucleolar structure. As a result of this different disposition of the chromatin, quail and chick cells, experimentally associated in chimeras, can be easily distinguished.

By the use of heterospecific combinations between quail and chick thymic rudiments, the respective contribution of endodermal epithelium, thymic mesenchyme, and blood-borne extrinsic elements to the histogenesis of the thymus has been studied. In the same way, it has been possible to investigate the evolution of the thymic lymphocyte population and to follow the chronology of stem cell inflows in the thymus during embryonic life.

Materials and Methods

White Leghorn chick and Japanese quail embryos were used. The eggs were incubated at 38° ± 1 C. The timing of the embryos was determined according to the number of somites in the young stages and to the length of the incubation period later on. In some experimental series, staging was done according to the developmental stages defined by Hamburger and Hamilton (30) for the chick and by Zacchei (31) for the quail.

Grafting techniques

Grafts of Thymic Primordia

GRAFT OF QUAIL THYMIC ENDODERM INTO THE CHICK SOMATOPLEURE. The lateroventral wall of the embryonic pharynx from quail embryos at 15- to 30-somite stages is treated by a solution of trypsin at 0.1% in Ca^{2+}, Mg^{2+}-free Tyrode for 20–30 min at 2°C. Trypsin digestion makes it possible to separate the endoderm pure from pharyngeal mesenchyme. The endodermal area which is selected for the graft at different developmental stages is represented in Fig. 1.

The endoderm is introduced in a slit made in the somatopleure of a 3- to 3.5-day old chick between anterior and posterior limb buds according to a technique previously described (32) (Fig. 2). The tissue should not be introduced in the coelomic cavity but left in contact with somatopleural mesenchyme which will participate to thymic histogenesis. If the explant falls into the coelome, it remains purely epithelial; missing the stimulating influence exerted by mesenchyme on its growth and differentiation, it degenerates and fails to give rise to thymic tissue (33). When the endoderm grows in contact with somatopleural mesenchyme, its differentiating capacities are expressed and it gives rise to the normal derivatives of the third, fourth pharyngeal pouches, and medioventral endoderm of the pharynx, involved in the graft (Fig. 1). Thus, in addition to thymic tissue, parathyroid, ultimobranchial body, and digestive tube are found developing in the body wall of the host. In heterospecific associations between quail endoderm and chick somatopleure, all these organs are chimaeric, their mesenchymal component being derived from the chick somatopleure. The host embryo is sacrificed 12 days after the implantation and the region of the body wall containing the grafted tissue is fixed for histological examination.

GRAFT OF COMPLETE THYMIC RUDIMENTS (ENDODERM AND MESENCHYME). Thymic primordia of various ages were grafted from quail into chick or reversely. At the early stages of development the area (presumptive or true) of the 3rd and 4th branchial pouches and arches, i.e. endoderm, mesenchyme and ectoderm, is dissected and grafted (Fig. 3). From the 6th day of incubation, the thymic rudiment can be distinguished along the jugular vein and isolated by careful dissection. In this experiment, the thymic endoderm being associated to its homologous mesenchyme is able to develop in the coelomic cavity, but a better evolution of the grafts is observed in the somatopleure, so that this technique was extended to the entire thymic primordium. The graft is made into the somatopleure of
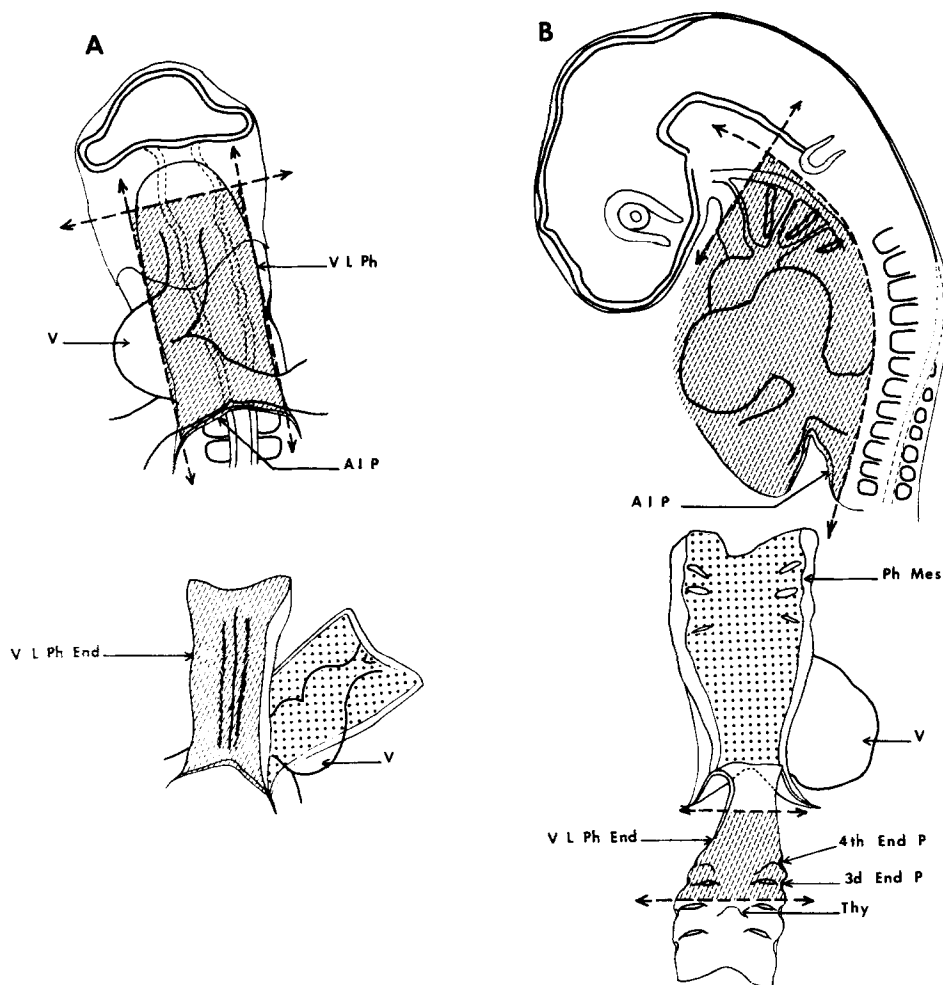
Fig. 1. Schematic representation of the endodermal area taken from quail embryos at various stages of development involving the presumptive thymic epithelium. (A) In 15-to 22-somite embryos the ventrolateral wall of the pharynx (V L Ph) is cut and the pharyngeal endoderm (V L Ph End) is isolated by trypsinization of the cardiac fold and grafted into the somatopleure of a chick embryo. (B) In older embryos a piece of the ventrolateral endoderm including the 3rd and 4th branchial pouches (3d, 4th End P) is selected for the graft. *AIP*, anterior intestinal portal; *V*, ventricle.

a 3- to 3.5-day old host. In some experiments the thymus is grafted on the chorioallantoic membrane (CAM) in 6-day host embryos according to a classical technique (34).

Grafts of Quail Rhombencephalic Neural tube into Chick Embryos

In order to study the participation of the mesenchyme of the 3rd and 4th branchial arches to thymus histogenesis, isochronic and isotopic grafts of quail neural rhombencephalon were carried out into chick embryos of the 6- to 9-somite stage. The neural crest origin of this mesenchyme has been demonstrated in this laboratory (35, 36).

The grafting technique previously described (26) is the following. In the first step the chick rhomb-encephalon is excised by microsurgery in ovo, endoderm, notochord, and somites being left in situ (Fig. 4). The quail neural tube and associated neural folds are isolated by incubation of the adequate
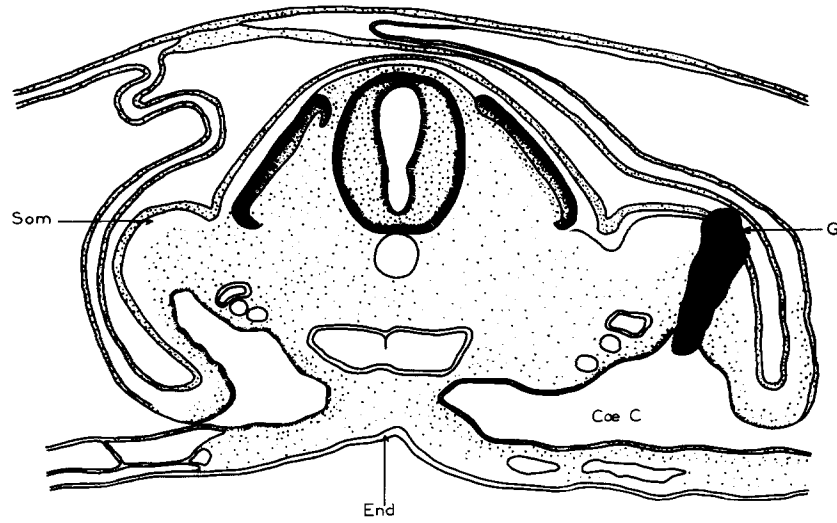
FIG. 2.   Transverse section in a 3-day old chick embryo which receives the graft (*G*) of a quail thymic endodermal rudiment in the somatopleure (*Som*). *End*, endoderm. *Coe C*, coelomic cavity.
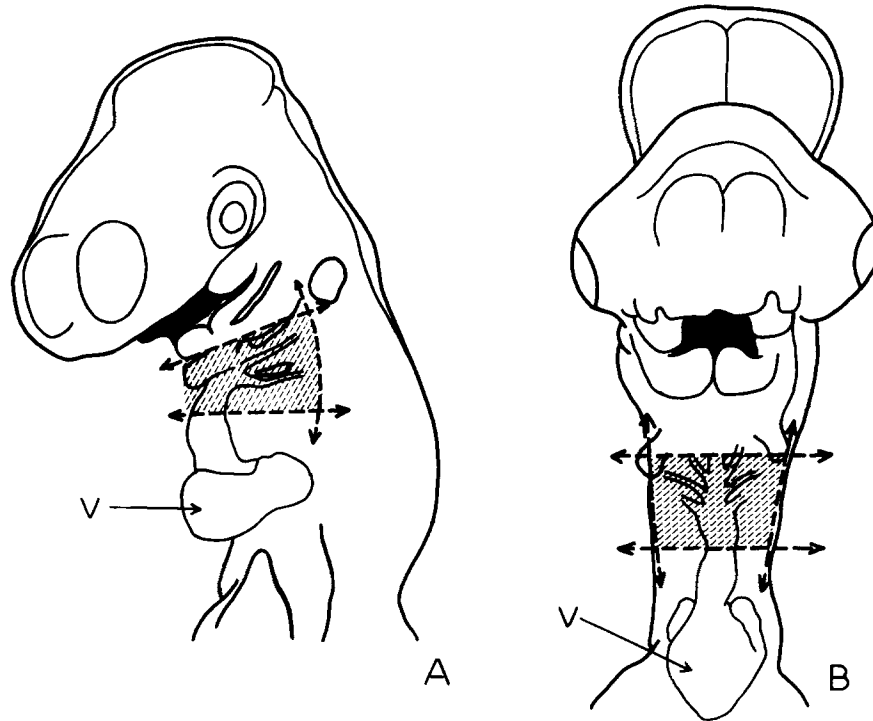

FIG. 3.   Schematic drawing showing the area taken in the quail at 3 to 4 days (A) and at 5 to 5½ days (B) of incubation involving the endomesodermal thymic rudiment. *V*, ventricle.
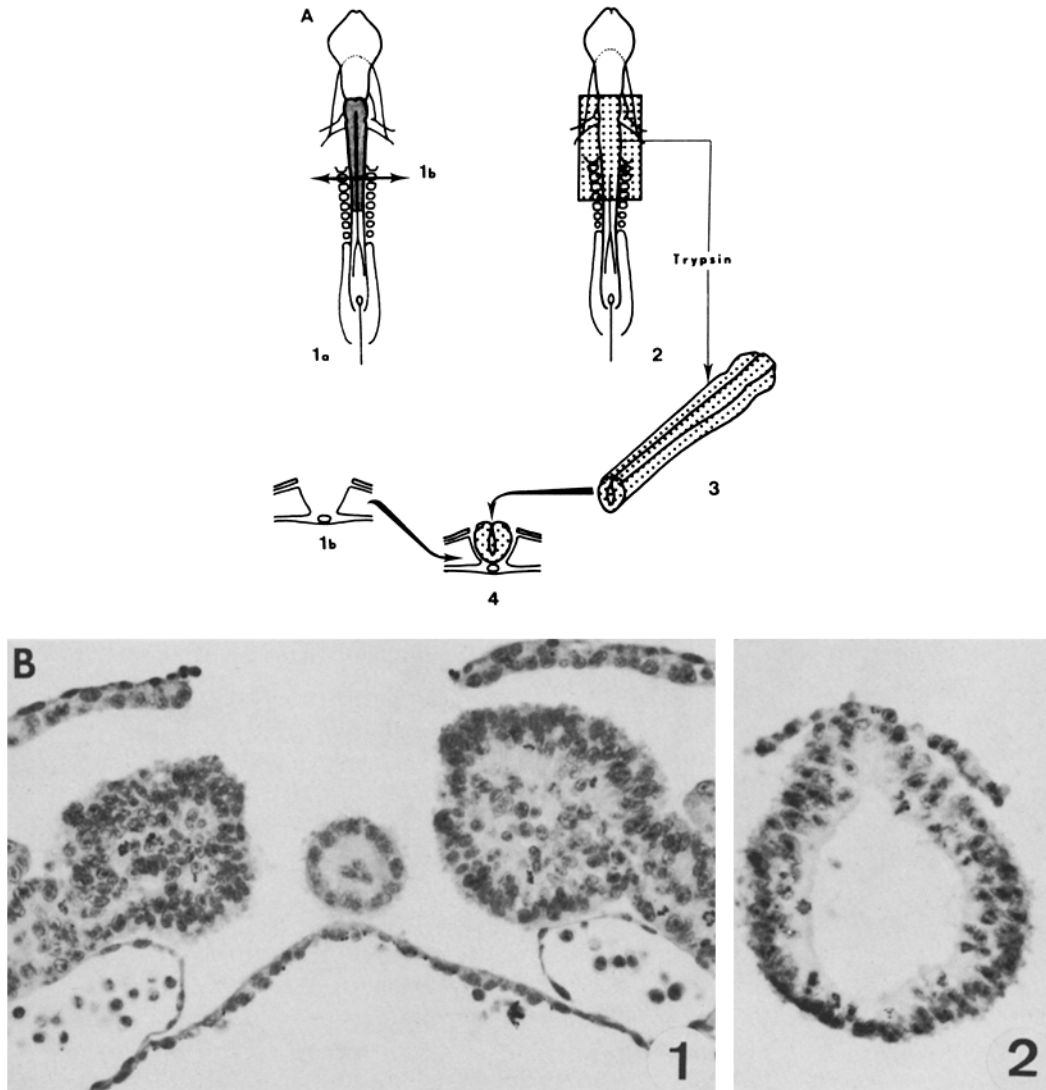
FIG. 4.   Isotopic and isochronic graft of a quail rhombencephalon into a chick embryo at 6- to 9-somite stages. (A) Schematic drawing of the experimental procedure: (1) the rhombencephalon (hachured area) is surgically removed from the chick host embryo. (a) dorsal view; (b) transverse section at the level of the excision. (2) the corresponding area is taken in a quail embryo at the same developmental stage and submitted to a solution of trypsin. (3) the quail rhombencephalon is isolated by trypsinization and in (4) grafted orthotopically into the chick host. (B) Transverse sections of the chick embryo (1) and of the isolated quail neural tube (2) before the graft. Feulgen-Rossenbeck. (1) × 310. (2) × 390.

transverse section of the quail embryo in Mg^{2+}, Ca^{2+}-free Tyrode solution additioned with 0.1% trypsin. Thus treated, the grafted neural anlage is perfectly devoid of mesenchymal cell contamination (Fig. 4). In the second step, the quail neural primordium is grafted into the chick host in the groove resulting from the excision of the hind-brain region. The grafted neural tube is rapidly

incorporated into the axial structures of the host and, when the graft has been successful, crest cells follow the normal migration pathways in the host tissues (37).

Histological techniques

Light microscopy. Chick and quail embryos were fixed in Zenker's fluid and their pharyngeal region was cut in 5μm transverse serial sections at the stages of 4 to 6th days of incubation to study the normal process of thymus histogenesis. At later stages the thymus alone was fixed and treated according to the same procedure. Sections were stained with hematoxylin and eosin. Sections of differentiated thymuses, from control and experimental embryos were treated according to the Feulgen-Rossenbeck staining procedure (38) for DNA, in order to distinguish quail from chick cell types. In an experimental series the panoptique technique according to Pappenheim (39) was applied in order to evidence basophilic cells in thymus rudiments. In some cases the same sections have been poststained by Feulgen-Rossenbeck's method. During this second treatment, the staining provided by the panoptique technique disappears and DNA-containing structures alone are stained. Thus, pictures of the same section stained with the two techniques may be compared in order to identify basophilic cells as belonging to the quail or chick species.

Electron microscopy. Normal and experimental thymuses were fixed in 6% glutaraldehyde in 0.1 M phosphate buffer, at pH 7.4 for 20 min at 4°C, and postfixed in 1% osmium tetroxyde in phosphate buffer for 1 h. Blocks were embedded in Epon and sections, stained with lead citrate and uranyle acetate, were observed in an Hitachi HS8 electron microscope.

Results

Comparative study of quail and chick embryonic thymus

Morphological Distinction of Chick from Quail Thymic Cell Types. Like in other tissues, the distinction between chick and quail cells in the thymus is based on the appearance of the interphase nucleus (Fig. 5).

LYMPHOID CELLS. In the quail thymus, cells with large nuclei are often encountered especially in the cortical area. Stained with the panoptique technique, these cells have a strongly basophilic cytoplasm. They are considered by several authors (see 4 for references) as the stem cells of lymphoid elements. Their nucleus shows a large central mass of heterochromatic DNA with irregular outlines (Fig. 6 a). At the electron microscope (EM) level, the nucleolus is made up of closely intermingled DNA- and RNA-containing structures, and corresponds to the type III previously described in quail cells (27, 40). In the chick, corresponding cells show a large, weakly stained nucleus where a thin Feulgen-positive ring of perinucleolar DNA can sometimes be distinguished (Fig. 6 b).

In quail lymphocytes, each nucleus contains one large and several smaller heterochromatic patches often attached to the nuclear membrane (Figs. 5–6a), while chick lymphocytes are characterized by several small strongly Feulgen-positive chromocentres dispersed in the nucleus (Figs. 5–6 b). At the EM level the differences are also very striking. Numerous, dense chromatin patches appear distinctly in large, medium, and small lymphocytes of the chick (Fig. 7). In the quail, a large heterochromatic condensation is present in all lymphocyte types (Fig. 8). In some sections of quail lymphocytes, the central DNA mass appears as a thick dark ring surrounding a clear central core (Fig. 9). Serial sections show that the heterochromatic mass is uniformly composed of densely coiled fibrillar DNA. The Feulgen-positive chromatin patches distributed on the nuclear membrane are also evident in electron microscopy (Fig. 8).

RETICULAR AND CONNECTIVE TISSUE CELLS. In the quail thymus, reticular cells,

numerous in the medulla and at the periphery of cortical lobes, are characterized by a single strongly Feulgen-positive centronuclear condensation (Figs. 5-6 a). Apart from this central mass, the nucleoplasm is only weakly stained by the Schiff reagent. In the chick, the nucleus of reticular cells shows a network of lightly stained chromatin. The same characteristics are found in connective cells, the nuclei of which is often elongated (Fig. 6 b).

Chronology of Thymic Development in Quail and Chick Embryos. Preliminary to the experimental study of thymus development in interspecific combinations, the chronological and morphological evolution of thymic primordium was compared in quail and chick. Our observations on the chick are in general agreement with the results reported by previous authors (5, 9, 10); the stages of significant events of thymus histogenesis in the two species are reported in Table I.

This comparative study makes it clear that successive steps of thymic histogenesis—formation of epithelial cords, lymphoid differentiation—occur a little earlier in quail than in chick, but according to a similar pattern. Thus, considering the close taxonomic vicinity of the two species one can assume that interspecific combinations of thymic rudiments can give rise to normally organized thymuses.

Experimental analysis of thymus development using quail-chick combinations

Through various tissue combinations selective quail nuclear labeling was achieved, affecting one cell category at a time: (a) the endodermal derivatives, (b) the mesenchymal component, (c) the lymphoid cell population.
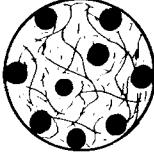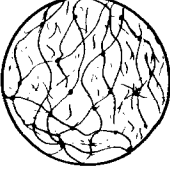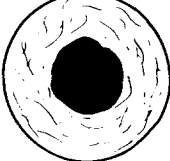
Graft of quail presumptive thymic endoderm into the chick embryo. Presumptive thymic endoderm from 15- to 30-somite quails was grafted in the somatopleure of 3-day chick embryos (Figs. 1 and 2). 12 days after implantation, the graft was observed histologically. It has been previously demonstrated in homospecific associations (33) that the thymic endoderm is determined early and differentiates into thymic tissue when associated to the somatopleural mesenchyme. The thymus developed in these conditions has a normal histological structure with cortex and medulla (Fig. 10). The whole lymphoid population is of chick host origin while reticular cells show the quail nuclear marker (Fig. 11). The perivascular endothelium and connective tissue cells belong to the chick, and they are derived from the somatopleural mesenchyme.

These results show that thymic endoderm isolated as early as the 15-somite stage is able to develop into thymic reticular cells and to induce the heterologous mesenchyme of the somatopleure to participate in thymus histogenesis. On the other hand thymus endoderm does not give rise to lymphoid cells, which in this experiment come from the host. They could derive either from thymic mesenchyme (i.e., the thymic pharyngeal mesenchyme in normal development or the heterologous somatopleural mesenchyme in the experiment reported above), or from extrinsic hemocytoblasts. The following experiments were devised to decide on this issue.

Role of the mesenchymal component in thymus histogenesis

GRAFT OF COMPLETE QUAIL THYMUS RUDIMENT [ENDODERM AND THYMIC PHARYNGEAL MESENCHYME] INTO THE CHICK EMBRYO. The total thymic anlage was taken from

NUCLEAR CHARACTERISTICS OF THE CELL
TYPES IN QUAIL AND CHICK THYMUSES

	Lymphocytes	Reticular cells	Connective cells
CHICK	numerous small clumps of chromatin	Evenly dispersed network of chromatin	
QUAIL	one large clump of heterochromatic DNA + small masses attached to the nuclear membran	One large central mass of heterochromatic DNA	

FIGS. 5-6. Schematic drawings (5) and micrographs (6 a and b) showing the nuclear feature of quail and chick thymic cell types after Feulgen-Rossenbeck staining. In the quail (6 a) the lymphocyte (L) nuclei contain one large heterochromatic mass and several smaller ones attached to the nuclear membrane. In the chick (6 b) several small chromocenters are dispersed in the nucleoplasm.

In the quail, the lymphoblasts (La) show a large nucleus with a Feulgen-positive centronuclear condensation while in the chick the nucleus is only clearly stained. Reticular (R) and connective cells of quail have usually one single heterochromatic mass. In the same cell types of the chick the nucleus contains a fine network of evenly distributed chromatin. 6 a × 1,450; 6 b × 1,300.

15-somite to 4-day quail embryos and grafted into the somatopleure of 3-day old chick hosts as previously described. The implant was fixed when the total age of the thymus was about 14 days. If lymphoid cells derive, even partially, from thymus mesenchyme proper, lymphocytes will have the quail nuclear marker. Actually, all the lymphoid cells of the thymic tissue came from the chick host, just as in the first experimental series, when the thymic endoderm was grafted alone. Reticular cells were of quail origin (Fig. 12) and connective tissue cells were of both donor and host type, showing that the somatopleural mesenchyme participates in the histogenesis of the explant. In the cortex, typical reticular cells characterized by long cell processes, showed the quail DNA rich nucleolus. Large, medium, and small lymphocytes as well as lymphocytoblasts on the contrary had the nuclear features of the chick (Fig. 12).

This experiment demonstrates that the lymphocytes derive solely from extrinsic elements absent from the quail thymic rudiment up to the 4th day of incubation. Neither endoderm nor mesenchyme of the thymic primordium have the ability to differentiate into lymphocytes. Moreover, these results show that the presumptive thymic endoderm selectively attracts the extrinsic lymphoid stem cells, even when grafted in an heterotopic location.
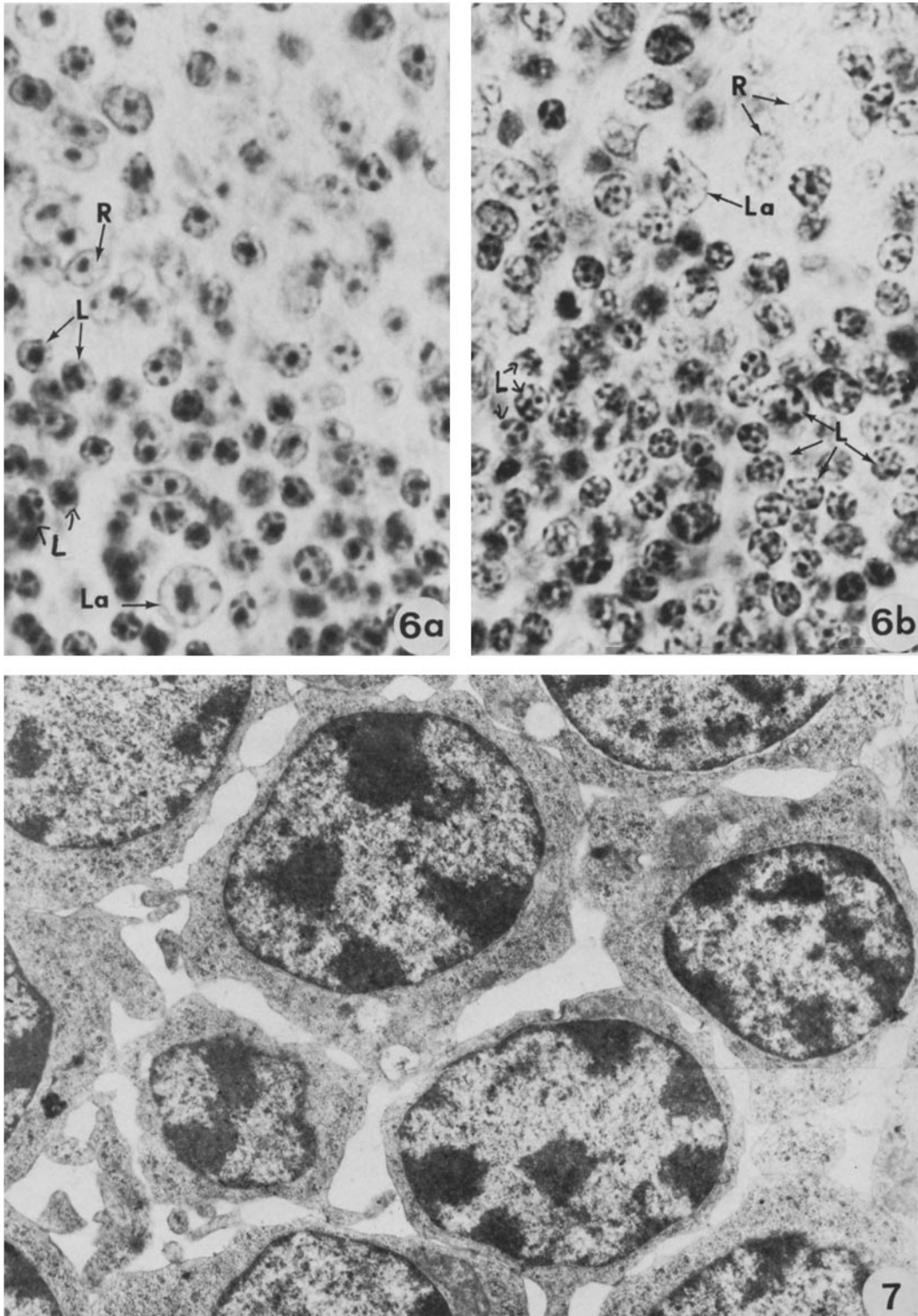
FIG. 6. See legend for Figs. 5-6.

FIG. 7. Electron micrograph of a chick thymic cortical area at 17 days of incubation. Several masses of DNA are seen in lymphocyte nuclei. Uranyle acetate-lead citrate. × 10,500.

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FIGS. 8-9.   Electron micrograph of the cortex in 16-day old quail thymus.
FIG. 8.   Small lymphocytes showing a large centronuclear DNA mass and several smaller ones attached to the nuclear membrane. Uranyl acetate-lead citrate. × 10,500.
FIG. 9.   Medium and large lymphocytes in which the centronuclear heterochromatic mass shows a clear central core. Uranyle acetate-lead citrate. × 10,500.

TABLE I

Occurrence of the Main Developmental Events During Thymic Histogenesis in Quail and Chick

|	Isolation of epithelial thymic rudiment from the embryonic pharynx	Fusion of thymic rudiments III and IV	First appearance of basophilic cells in the thymic epithelial cord	First appearance of lymphocytes in the thymic rudiment	Beginning of lobe formation	Lobulation of thymic lobes	Appearance of vascularisation in the epithelio-mesenchymal thymus anlage	Completion of lobe formation
Quail Stages of development according to Zacchei (31)	St. 19–20 (4½–5 days of incubation)	St. 21 (5 ½ days of incubation)	St. 20–21 (5–5 ½ days of incubation)	8 days of incubation	7 days of incubation		9 ½–10 days of incubation	9–10 days of incubation
Chick Stages of development according to Hamburger and Hamilton (30)	St. 25 (4 ½–5 days of incubation	St. 28 (5 ½–6 days of incubation	St. 29–30 (6 ½ days of incubation)	9 ½ days of incubation	≈8 days of incubation	8 days of incubation	8 days of incubation	15 days of incubation

STUDY OF THE EVOLUTION OF THE THYMIC MESENCHYMAL COMPONENTS BY SELECTIVE LABELING OF BRANCHIAL ARCHES MESENCHYME. It has been previously shown that the mesenchyme of the branchial arches derives entirely from the neural crest except for the muscle plate which is of mesodermal origin (35–36). Isotopic and isochronic grafts of a quail rhombencephalic primordium into a chick embryo at the 6- to 9-somite stage result in the invasion of the 3rd and 4th branchial arches of the chick host by quail cells (Fig. 13). The thymic rudiment is chimeric from its first developmental steps: the host endodermal pouch is surrounded by quail mesenchymal cells; later on, the chick thymic epithelial cord is surrounded by a thin capsule of quail mesenchyme (Fig. 14). Thereafter, the invasion of the epithelial anlage by connective cells may be followed and the contribution of mesenchymal elements to the differentiated thymic tissue determined. Mesenchymal cells follow the blood vessels which penetrate into the cortex and the medulla, the endothelium of which is of host origin (Fig. 15). The thymic rudiment is invaded by capillary buds of mesodermal origin while thymic connective cells derive from mesenchyme of neural crest origin. In none of the 40 cases of differentiated thymuses observed in this experimental series did the lymphocytes show the quail nuclear marker. This experiment confirms the previous result, i.e., that the branchial arch mesenchyme which participates to thymic histogenesis does not give rise to lymphoid cells. Colonization of the thymus rudiment, by extrinsic hemocytoblasts, being established the next experimental step, was to study the timing of this colonization in quail and chick.

Timing of Colonization of the Thymic Rudiment by Lymphoid Stem Cells. Quail thymic primordia of various ages from 4 to 8 days of incubation have been grafted into the somatopleure of 3-day chick embryos and fixed when their total age (age at the time of the graft and duration of the graft) reached 14 days. The reverse graft of chick thymic anlage from 4- to 9.5-day embryos into 3-day quails has also been carried out in the same conditions. The lymphoid population of the thymuses which develop in those conditions should be of host origin when they have been taken before the normal time of stem cell invasion. On the contrary, if
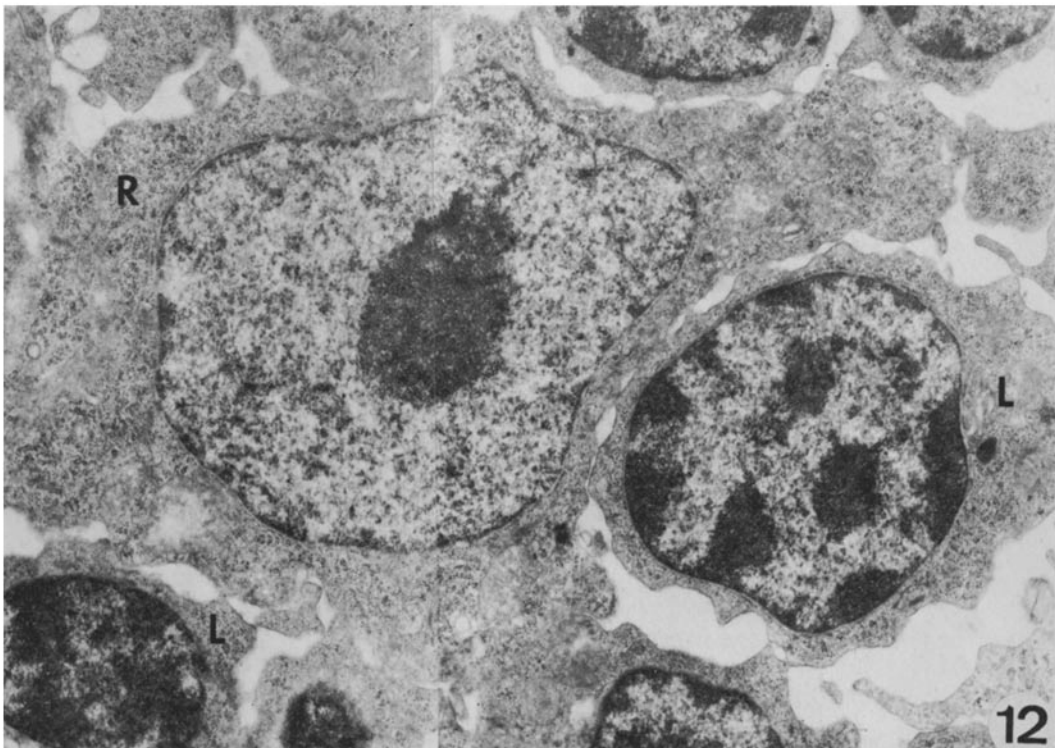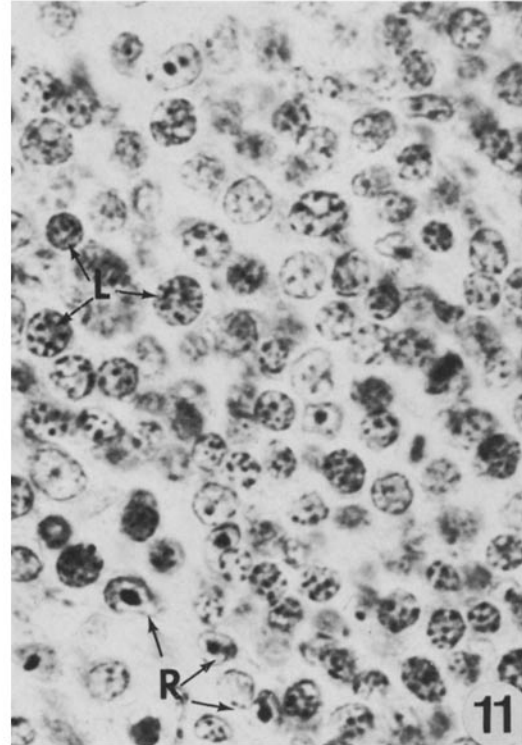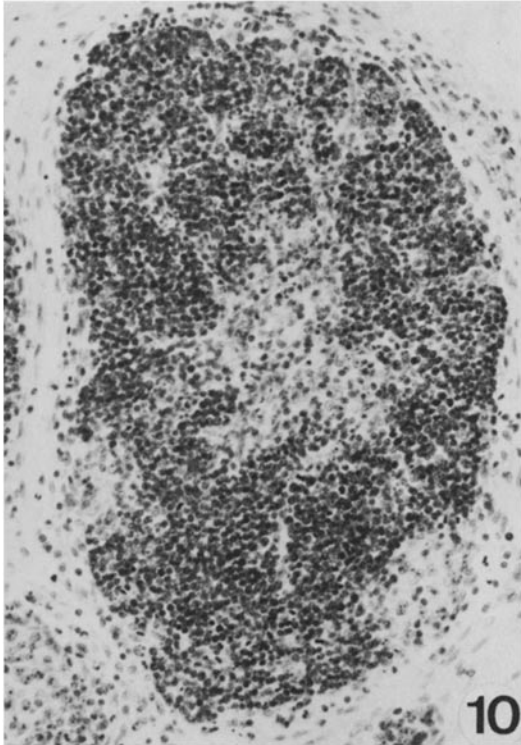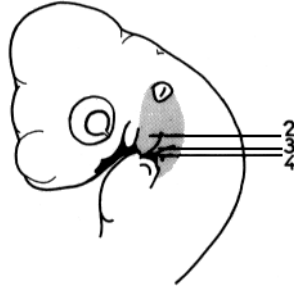
FIG. 13. Schematic drawing of a chick embryo which has received at 6- to 9-somite stage the graft of a quail rhombencephalon. The hachured area shows the region where the neural crest cells originating from the graft have migrated forming the mesenchyme of the 2nd, 3rd, and 4th branchial arches.

they are taken after the stem inflow, donor lymphocytes will be found in the organ. Moreover, through evaluation of the proportion of host and donor lymphoid cells, whether the inflow of stem cells is a permanent or a transitory process during thymus organogenesis may be determined. If a single, transitory inflow of stem cells occurs at a precise moment of thymic histogenesis, thymuses taken from the donor after the end of this process will contain only their own lymphocytes. The results obtained in this experiment are reported Table II. In the first series, i.e. transplantation of quail thymus rudiments into chick, the lymphoid cells are entirely of host origin in the implants coming from 4-(96 h)- and 4-5-(118 h)-day old quail embryos [stages 18 and 19 of Zacchei (31)]. If the rudiments are taken at 5 and 5.5 days [stages 19-20 of Zacchei (31)], they contain both quail and chick lymphoid cells when they have reached the total age of 14 days in graft. In certain areas of the explant, cells of one of the species can be represented alone, in most sections however a mixture of quail and chick lymphocytes is observed.

When the thymuses are taken from 6-(144 h), 7-, or 8-day old quail embryos the lymphocytes which they thereafter contain were all, or nearly all, of quail origin. In the reverse associations, that is grafts of chick thymuses into quail embryos the following results were obtained: chick thymuses taken from 5- to 6-5-day old embryos (stages 26 to 29 of ref. 30) contained exclusively host lymphoid cells when they had reached the total age of 14 days at the end of the graft. Thymuses taken from 6-5 to 8-day chick (stage 30 to 34-35 of ref. 30) contained a mixture of quail and chick cells (Fig. 16) while their lymphoid population was of donor origin when they originated from embryos of 8 days onwards.

FIG. 10. Chimeric thymus resulting from the graft of a thymic endodermal rudiment of a 16-somite quail embryo into the somatopleure of a 3-day old chick .13 days after grafting. Feulgen-Rossenbeck staining. × 240.

FIG. 11. Detail of the precedent section showing that the lymphocytes (L) belong to the chick species and the reticular cells (R) to the quail. Feulgen-Rossenbeck staining. × 1,100.

FIG. 12. Cortex of a chimeric thymus resulting from the graft of the endomesodermal thymic rudiment of a 30-somite quail into a 3-day old chick 14 days in graft. R, reticular cell showing the large DNA-rich nucleolus of the quail. L, typical chick lymphocytes. Uranyle-acetate-lead citrate. × 10,500.
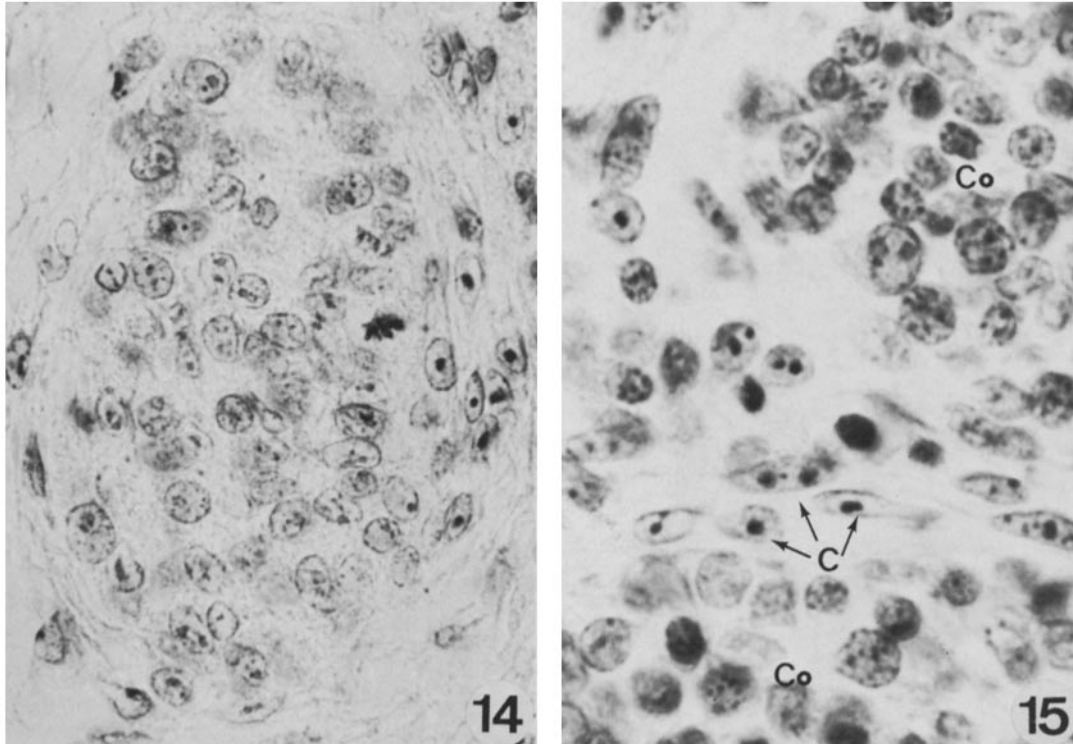
Fig. 14. Thymic rudiment in a 7-day old chick embryo after the graft of a quail rhombencephalon according to the experimental procedure indicated Fig. 4. The epithelial thymic anlage is made up of chick cells while the thymic mesenchymal capsule belongs to the quail species. It originates from the rhombencephalic neural crest which gives rise to the mesenchyme of the 3rd and 4th branchial arches. Feulgen-Rossenbeck. × 850.

Fig. 15. Same experiment as in Fig. 14. Section of the thymus at 13 days of incubation. The connective cells (C) lining the blood vessels derive from the quail thymic mesectoderm. Thymic cortex (Co) shows reticular cells and lymphocytes of host origin. No lymphocytes show the quail nuclear marker and then no lymphocytes derive from the mesodermal thymic component. Feulgen-Rossenbeck. × 1,450.

These experiments indicate that stem cells of lymphocytes are present in thymus anlage from the stages of 5 days in quail and 6.5 days in chick embryos. This stage coincides with the time at which basophilic cells become detectable in the thymus in both species (Table I). It seemed interesting to find out whether these cells are the precursors of thymus lymphocytes and therefore if in our experimental conditions they show the nuclear characteristics of the host in the grafted thymus. 6-day old chick thymic rudiments were grafted for 3 to 4 days into 3-day old quail embryos and then fixed in Zenker's fluid and treated according to the Pappenheim's and Feulgen-Rossenbeck's associated techniques. In these conditions the epithelial chick thymic rudiments contained a number of cells with a strongly basophilic cytoplasm showing also the quail marker (Fig. 17). The hypothesis of Moore and Owen (24) that the first detectable lymphoid elements of the embryonic thymus are cells with a large nucleus and a strongly basophilic cytoplasm is thus confirmed.

TABLE II

Time of Colonization of Thymic Rudiment by Blood-Borne Stem Cells

Stage of donor embryo at time of graft	Days of incubation	Type of lymphocytes in 14-day old grafted thymus		
		Donor type	Host + donor type	Host type
Quail				
St. 18*	4			4/4
St. 19-20	4 ½-5			3/3
St. 20-21	5-5 ½		3/6	3/6
St. 22	6	4/4§		
Chick				
St. 29‡	6-6 ½			4/4
St. 29-30	6 ½		4/4	
St. 30	6½-7		4/4	
St. 30-31	7	4/7	3/7	
St. 33-34	8	7/9	2/9	
St. 35	8 ½-9	5/5		

* According to Zacchei (31).

‡ According to Hamburger and Hamilton (30).

§ Number of positive results on number of embryos observed.

Moreover, it can be concluded that in both species at a precise stage of its histogenesis the thymic rudiment receives a rapid inflow of stem cells the duration of which is relatively short, i.e. about 24 h in the quail (during the 6th day of incubation) and 36 h in the chick (during the second half of the 7th day and the whole 8th day of incubation). After this stage penetration of stem cells in the thymus is extremely slow if not totally nonexistent.

Further problems arise: does the thymus cease to be attractive for stem cells after this stage, or are thymic hemocytoblasts unavailable in the embryo during a certain period of embryonic life? On the other hand, does the initiation of stem cell inflow into the thymus depend either on the apparition of its capability to retain the hemocytoblasts or on the stage at which those cells become available in the embryo? Toward solving these problems, stem cells with a capacity to differentiate into thymic lymphocytes were searched for in the embryo at stages earlier and later than that of normal thymic invasion.

Presence of Stem Cells in the Embryo at Various Stages of Development. In order to know whether thymic lymphocyte stem cells are present in the embryo before the stage when they normally participate in thymus histogenesis, the following experiment has been carried out: thymic rudiments were dissected from 4-day quail embryos, grafted for 2 days in 3-day old chick somatopleure and then retransplanted into a 3-day old quail for 8 days (Fig. 18). At this time the quail thymus aged 14 days total was observed histologically after Feulgen-Rossenbeck staining.

In this case the lymphoid population of the thymic tissue showed the characteristic nuclei of the chick while reticular and connective cells belonged to
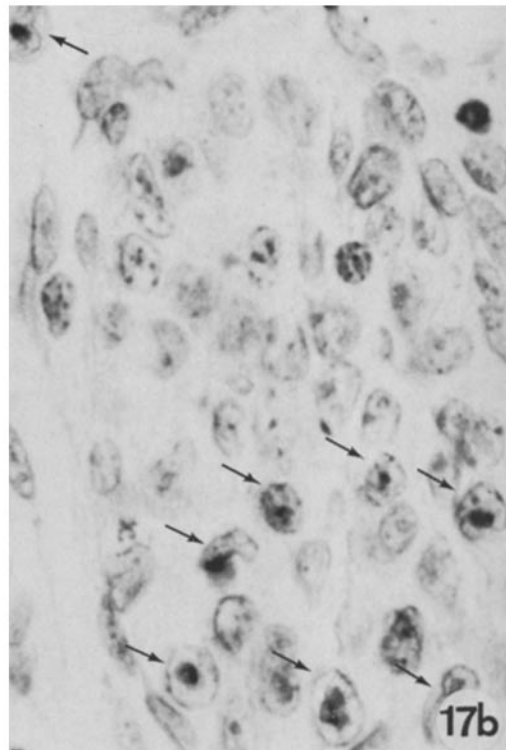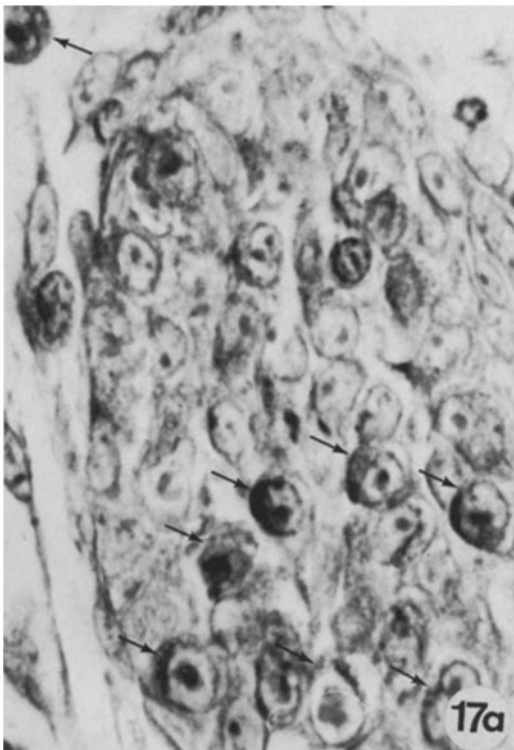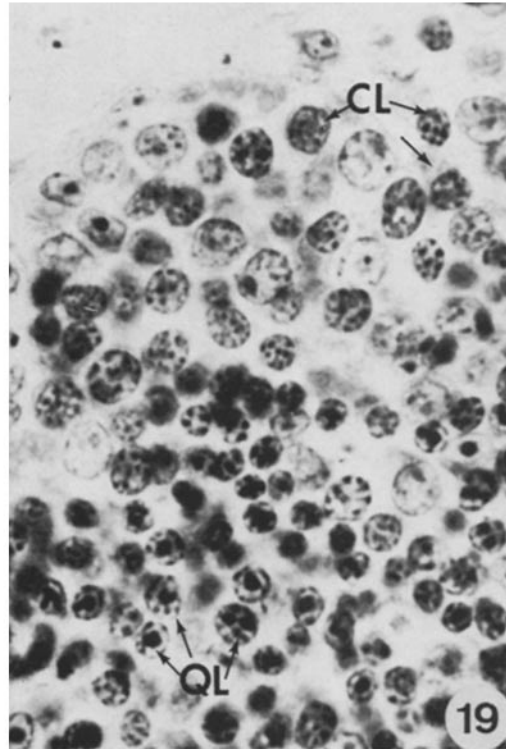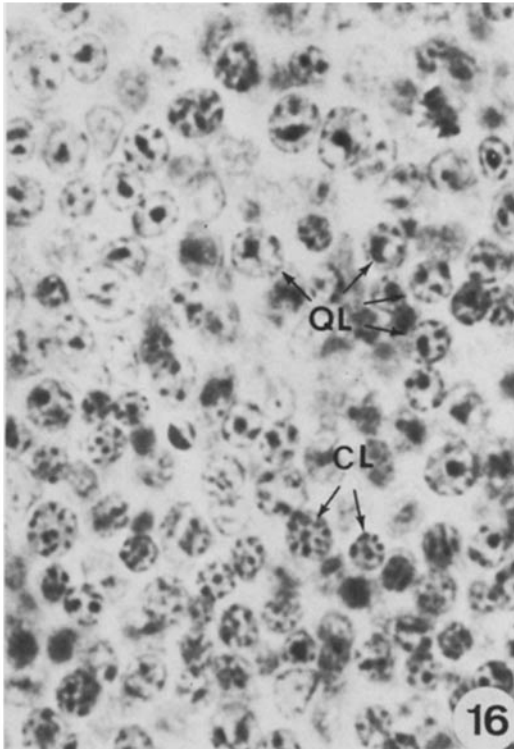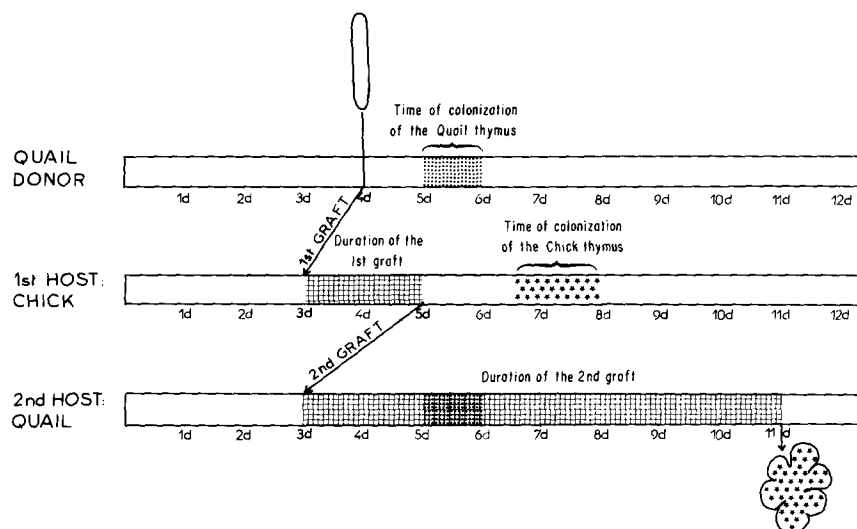
Fig. 18. Diagram of the experimental procedure devised to test the availability of hemopoietic stem cells in the chick embryo before the normal time of thymic colonization. A quail thymic rudiment from a 4-day old embryo is grafted into a 3-day old chick for 2 days and then transplanted into a 3-day quail host embryo. When the total age of the grafted thymus reached 14 days it is observed histologically and shows a lymphoid cell population entirely of chick origin. This demonstrates that the stem cell inflow into the quail thymus occurred exclusively during the first transplantation in the chick host.

the quail species. These results show that hemocytoblasts able to respond to thymic endoderm attraction and induction are available in the embryo as soon as the 4th day of incubation that is before the stage at which they normally enter the thymus primordium. This experiment also confirms that the duration of the first inflow of cells into the thymus is limited, since none or very few quail cells were found in the thymus after the second transplantation in quail embryo.

In a second experimental series thymic rudiments were taken from 4-day old quail embryos and grafted on CAM of 8.5-day old chicks for 10 days in order to see whether thymic hemocytoblasts are present in the blood of the chick embryo after the stage of thymus colonization. These grafts showed a normal development of thymic tissue in which lymphoid cells were of host origin.

Those experiments demonstrate that stem cells competent to respond to thymic attraction are present in the embryo before and after the stage at which

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Fig. 16. 6-day old chick thymus grafted for 8 days in a quail embryo. Mixture of quail (QL) and chick lymphocytes (CL). Feulgen-Rossenbeck. × 1,280.

Fig. 17. 6-day old chick thymus grafted for 4 days in a quail embryo. (a) Panoptique technique showing basophilic cells (arrows). × 1,280 (b) The same section is post-stained with Feulgen-Rossenbeck technique. The basophilic cells show the quail nuclear marker. This demonstrates that the hemopoietic cells of the thymus have an extrinsic origin. × 1,280.

Fig. 19. Thymus of a 8-day old quail embryo grafted for 10 days into a chick host. In the external region of the cortex quail lymphocytes have been replaced by host ones (CL). Quail lymphocytes are present in the internal region of the cortex (QL). Feulgen-Rossenbeck. × 1,140.

they colonize the thymus. They penetrate into the thymic rudiment at a precise stage of its organogenesis and during a limited period of time, after which none or a very small inflow of cells takes place.

During the colonization period, the thymic rudiment attracts and retains blood-borne stem cells. When a certain amount of basophilic cells are present in the epithelium the latter loses at least partially its capacity to retain more circulating hemocytoblasts.

The next problem is to know how long this nonattractivity or low attractivity period of the embryonic thymus lasts. In previous experiments the graft period ended when the total age of thymic rudiment was 14 days. The heterospecific implantation time was protracted in order to see whether a new inflow of stem cells occurs during embryonic life.

Renewal of Lymphoid Stem Cells in the Thymus at the end of Embryonic Life. Thymic rudiments of 8- to 10 day old quail embryos were grafted into the somatopleure of 3-day chick embryos for 8 to 15 days. The proportion of chick and quail lymphocytes in the grafted thymus was evaluated by counting in each case about 2.500 cells at four different levels of the thymus, in the external and internal cortex. The results (Table III) show that a complete renewal of the

TABLE III
Percentage of Chick Lymphocytes in Quail-Grafted Thymuses

Age of thymus at time of graft	Duration of the graft																	
	7 days		8 days		9 days		10 days		11 days		12 days		13 days		14 days		15 days	
	EC*	IC	EC	IC	EC	IC	EC	IC	EC	IC	EC	IC	EC	IC	EC	IC	EC	IC
7 days	0	0	5,5	0			99	93	69	35	98	93	100	93	83	54	100	100
8 days	0	0	0	0	10	2,5	83,5	5,5	87	70,5	55	28	99,5	81	99	99	100	100
9 days	0	0	0	0	38	2	24	7	51	15	83,5	56,5	86	45	100	98,5	100	100
10 days	0	0	10	0	56	5	75	26	98	91	96,5	81	99,6	95,9	100	97,5	100	100

* Two cases were observed for each experiment. EC, external cortex; IC, internal cortex.

lymphocyte population occurs during the experiment. After 8 days in graft no host lymphocytes were present in the quail thymus: only scattered lymphoblasts could be detected in the peripheral cortex area. Chick lymphocytes first appeared in the external region of the cortex of the grafted thymus and progressively extended into the deeper cortical layers and then into the medulla. After 15 days in the chick, whatever the age of the thymus at grafting, practically the whole lymphoid population of the implant was of host origin in the 18 cases observed.

The reverse graft of thymus from 9- and 10-day old chick into 3-day quail embryos was carried out. The duration of the graft was of 8 and 11 days. The results obtained were in agreement with the previous ones. A progressive renewal of donor by host lymphocytes occurred in the grafted thymus. Thus, the thymus is the site of successive waves of lymphoid stem cells which completely renew the dividing lymphoid cell population of the organ.

Discussion

Our results confirm the findings of Moore and Owen (23, 24) and Owen and Ritter (25) about an extrinsic origin of the lymphoid stem cells of the thymus. By interspecific combinations performed at early developmental stages, selective labeling of either thymic endoderm or thymic mesenchyme showed that none of these rudiments are able to differentiate into lymphocytes. From evidence of the nuclear structural differences in the quail and in the chick, interspecific graft of early thymic rudiments has made it possible to visualize the "homing" of lymphoid cells into the developing thymus, and to appreciate through simple histological observation the proportion of host and donor lymphocytes at the successive times of grafting experiments. Chromosomal markers previously used (23–25) give informations only on dividing cells, while the "quail-chick system" covers the great majority of the cellular population at a given time, that is the nondividing cells.

The correctness of the assumption that the lymphoid precursor cell (24, 25) is the large basophilic cell seen in the thymic rudiment was demonstrated unquestionably in this work. It was possible to follow the first inflow of quail basophilic stem cells and their subsequent differentiation into lymphocytes in a chick thymic anlage grafted into a quail embryo.

It appeared that the thymus is the site of production of successive waves of lymphocytes originating from a pool of stem cells which is completely renewed once during the time of embryonic and early postnatal life. A first inflow of stem cells occurs at a precise stage of thymus organogenesis and lasts about 24 h in the quail and 36 h in the chick. Afterwards, thymic tissue attracts very few if any hemocytoblasts during a period of a few days until another inflow begins and new stem cells appear in the peripheral cortex. They start to divide while lymphocytes originating from the first stem cell wave leave the thymus. Lymphocytes from the second stem cell inflow progressively invade the whole thymus, from the external to the internal cortex and the medulla. It seems from our experiments that the whole lymphoid population of the thymus is renewed around hatching time in the two species. Because the grafted thymus appeared perfectly healthy in all of our experiments we feel secure that the renewal of lymphoid stem cells that we observed is a normal process. It should be noted, however, that the timing at which this event normally occurs cannot be exactly determined in our experimental conditions because of the disturbance caused by adaptation of the tissue to graft conditions and because of the differential rates of development of the two species.

In addition, these experiments show that the heterotopic grafting of endodermal thymic primordium results in the differentiation of a normally organized thymus. As early as 15-somite stage, i.e. before formation of the 3rd and 4th branchial pouch, some cells of pharyngeal endoderm are determined to differentiate into thymic reticulum. However, this differentiation cannot occur if the thymic epithelium is not associated with a mesenchymal tissue as previously shown by Auerbach (18, 19) in the mouse. If it is true that the mesenchymal component of the thymus promotes epithelial morphogenesis, our experiments

show that, inversely, early determined thymic endoderm is able to induce an heterologous mesenchyme to participate in thymus histogenesis and to give rise to normally differentiated thymic tissue.

It is likely that the mechanism of stem cells' attraction by the thymus is due to a diffusible substance to which the hemocytoblasts of the circulating blood are sensitive. It is interesting indeed to notice that the penetration of hemocytoblasts into thymic epithelium occurs before the vascularization of the rudiment. Therefore, they must have migrated from the blood vessels into the surrounding mesenchyme and then penetrated the basement membrane. The fact that thymic endoderm, grafted in a heterotopic location, attracts lymphoid stem cells of the host suggests that the cell type responsible for the elaboration of the attractive substance is the epithelial reticulum. Quail-chick combinations of thymic rudiments performed in these experiments showed that the mechanism of attraction of lymphoid stem cells by the thymus is not species specific since stem cells of the chick can colonize the thymic reticulum of quail and inversely.

Because of the fact that after birth the thymus is the site of constant inflow of stem cells from the bone marrow [see Metcalf and Moore (4) for review], one can assume that the same mechanism of attraction is maintained in the adult organ. During embryogenesis, certain tissues apparently exert such an attraction on migratory cells. This seems to be the case both for the genital ridge which attracts and retains primordial germ cells (41, 42) and for the various organs colonized by neural crest cells during early embryogenesis [see Horstadius (43), Weston (44), Le Douarin (29) for review]. In all these cases the attraction is a transitory phenomenon which disappears during the process of organogenesis. The hemo-poietic organs seem to be an exception in this respect as they retain this capacity to attract stem cells from early histogenetic stages through embryonic and adult life.

Cell tracer techniques including sex chromosome marker systems and the use of tritiated thymidine have demonstrated both the existence and the importance of migration streams of blood-borne hematopoietic cells between different tissues in the course of the ontogeny of the hemopoietic system (see 4 for review). Moore and Owen (23, 24) using the sex chromosome marker system to trace cell migration in parabiotic chick embryos showed a chimerism in the population of dividing cells not only in the thymus, but also in the other hemopoietic organs, the bursa of Fabricius (45), the spleen,[1] and the bone marrow (23).

Using the quail-chick marker system it was possible to demonstrate that in these organs [spleen (46), bursa of Fabricius (47)], as in the thymus, hemopoietic differentiation is entirely dependent on blood-borne immigrant stem cells. Recent observations (Le Douarin and Jotereau, unpublished data) have revealed that the hemopoietic cells of the bone marrow do not derive from the in situ mesenchyme of bone rudiment and especially the periosteal mesenchyme as suggested by Bloom (48), but from immigrant cells carried via the circulation.

Thus, it appears that hematopoietic differentiation in the embryo employs a similar basic mechanism for all blood cell-forming organs. Very early in

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[1]Moore, M.A.S., and J.J.T. Owen. 1965. Unpublished observation quoted in Metcalf and Moore (ref. 4).

development, hemopoietic stem cells are carried by the blood. They are selectively and sequentially attracted and retained by the various hemopoietic tissues, onward from a precise stage of their development. The problem then arises concerning the primary source of these cells and of the onset of the initial commitment to respond to the specific attraction and induction exerted by each differentiating hemopoietic organ.

According to the yolk sac migration concept developed by Moore and Owen (23) and Metcalf and Moore (4) the stem cells entering the circulation and initially colonizing fetal liver in mammals and primary lymphoid tissue are yolk sac-derived. At later stages following decline in yolk sac hemopoiesis, the expanding stem cell population colonizes spleen and marrow which replace the yolk sac as a source of circulating stem cells for primary lymphoid tissue. Since the yolk sac is the first important site of hemopoietic activity in the embryo. This theory appears reasonable.

Until now this hypothesis has not been unequivocally demonstrated by experimental results, so that this question has remained a controversial subject (46, 49–51). Recent experimental evidence has been obtained by grafting quail embryos on chick yolk sacs at very early stages. Hemopoiesis in the spleen and thymus is always of the embryo type (quail), proving that in this case yolk sac stem cells are not involved in the differentiation of intraembryonic hemopoietic organs.

Summary

Differences in the structure of the interphase nucleus between two species of birds, the Japanese quail (*Coturnix coturnix japonica*) and the chick (*Gallus gallus*) has been used to distinguish cells from different origins in interspecies combinations. This biological cell marking technique was applied to thymus histogenesis. Using various combinations between components of quail and chick thymic rudiments, the respective contribution of endodermal epithelium, mesenchyme, and blood-borne extrinsic elements to the histogenesis of thymus was analyzed. It was demonstrated that the whole lymphoid population of the thymus is derived from immigrant blood-borne stem cells which are chemically attracted by the endoderm of the 3rd and 4th pharyngeal pouch. The latter is determined to differentiate into thymic epithelial reticulum as soon as the 15-somite stage, and is able to attract blood stem cells even when transplanted in an heterotopic position such as the ventral body wall of the embryo. It was shown that the thymic mesenchyme originates from the neural crest mesectoderm which colonizes early the 3rd and 4th branchial arches. It participates in the formation of perivascular mesenchyme, but does not give rise to lymphocytes.

From heterospecific transplantations of quail thymuses into chick embryo (and inversely) at various stages of development it appeared that the thymic rudiment becomes attractive for lymphoid stem cells at a precise stage of its evolution for each species. The attractivity period lasts about 24 h for the quail and 36 h for the chick. Then, the inflow of stem cells becomes very low until the end of the incubation period. At this time, a second wave of lymphocytoblasts invades the

thymus and the primitive embryonic lymphoid population is completely renewed around the hatching time.

Competent thymic stem cells are present in the blood before and after the period of physiological thymic attractivity. The identity of basophilic cells appearing in the thymus during its histogenesis and lymphoid stem cells has been demonstrated from the analysis of quail-chick chimeric thymuses.

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