Analysis of endoderm formation in the avian blastoderm by the use of quail-chick chimaeras

The problem of the neurectodermal origin of the cells of the APUD series

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SUMMARY

The formation of the endoderm has been investigated in chimaeric embryos resulting from the combination of the lower and upper germ layers taken from chick and quail embryos at stages 2–6 of Vakaët (1962). The ability to recognize quail from chick cells made it possible to follow the fate of each germ layer during development. It appeared that the primitive hypoblast participates in the formation of the anterolateral extra-embryonic endoderm while the embryonic endoderm is formed later by migration of cells of the ectomesoblast through Hensen’s node and the primitive streak.

Further interspecific combinations were carried out between ectoderm and endoderm + mesoderm from quail and chick embryos at stages 5–7 of Hamburger and Hamilton. The explants were grafted into chick embryos for several days and the intestinal structures which developed were observed. No contribution of cells from the neurectoderm to the endoderm was found. In contrast, cells coming from the neural crest colonized the intestinal structures and gave rise to the enteric ganglia. It was concluded from these observations that the enterochromaffin and endocrine cells of the gut epithelium do not originate from the neurectoderm.

INTRODUCTION

In the avian embryo, gastrulation begins during progression of the germ in the oviduct; when the egg is laid, the embryo is already composed of two layers, the so-called epiblast or ectophyll and hypoblast or entophyll. Hypoblast formation has been considered as occurring either by migration of individual cells from the margin of the blastoderm (Duval, 1884) or by delamination of the blastodisc (Peter, 1938).

At later developmental stages, when the formation of the lower germ layer becomes completed, the term hypoblast is replaced by either endoblast or endoderm, and the term epiblast is replaced first by ectomesoblast then by ectoblast or ectoderm.

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It has been clearly established by various cell marking techniques (vital dyes, carbon or ferritin marks) that migration of cells from the posterolateral region of the blastoderm towards its presumptive cephalic border significantly contributes to the formation of the endoderm (Pasteels, 1937; Malan, 1953; Bellairs, 1953). On the other hand, the participation of cells from the epiblast, through the primitive streak, to the developing endoderm was attested by experiments using isotopic labelling (Nicolet, 1965; Rosenquist, 1966). This result was confirmed by Vakaët (1970, 1974) using either time-lapse cinematography or the cell marking technique devised by one of us (Le Douarin, 1969, 1971, 1973a, b; Le Douarin & Rival, 1975). Concerning the fate of the hypoblast and its developmental relationships with the endoderm, Vakaët (1970) showed furthermore that the hypoblast slips below the upper layer contemporary with the formation of the endoderm.

Using the quail-chick marker system, we have investigated the problems of the developmental fate of the hypoblast and of the formation of the endoderm.

A related problem is a possible participation of the neural ectoderm in endoderm formation, as proposed by Pearse and his coworkers (Pearse, 1969; Pearse & Polak, 1971; Pearse, Polak & Bussolati, 1972; Pearse & Takor Takor, 1976). For these authors, the enterochromaffin and endocrine cells associated with the gut epithelium could be of neural crest origin. Many attempts have been made by our group to test this hypothesis, especially by following crest cell migration through interspecific grafts of neural primordia between quail and chick embryos (Le Douarin & Teillet, 1974; Le Douarin, 1974, 1975; Le Lièvre & Le Douarin, 1975). We have been able to demonstrate the neural origin of calcitonin producing cells which develop in the 5th pharyngeal endodermal pouch (Le Douarin & Le Lièvre 1970; Le Douarin, Fontaine & Le Lièvre, 1974; Polak et al. 1974), but we have never observed neural crest cell migration into the endoderm of the gut, whatever the level of the neural axis at which the graft has been done. Similar conclusions were drawn by Andrew concerning the enterochromaffin cells (1963, 1974) and by Andrew (1976) and ourselves (unpublished data) concerning the endocrine cells of the pancreas.

However, it was interesting to see whether at early stages cells could migrate from the ectoblast or from the neural plate and eventually participate in endoderm formation. Some of the experiments reported below have been devised to answer this question.

MATERIAL AND METHODS

The experiments were performed on White Leghorn chick (Gallus gallus) and Japanese quail (Coturnix coturnix japonica) blastoderms. The stages of the chick embryos were determined according to the nomenclature of Vakaët (1962) for early development and according to Hamburger & Hamilton (H. and H.) (1951) for the later stages. For the quail, whose development is faster, the stages were determined by analogy with the chick.
Two series of experiments were devised.

1. **Interspecific association of lower and upper germ layers of quail and chick blastoderms from stages 2 to 6 of Vakaët**

The stages 2–3 of Vakaët were reached after 5–8 h of incubation for the chick and 4–6 h for the quail. The hypoblast is made up of a monolayer of cells and the epiblast of a randomly arranged mass of cells thickened in the central area. Koller’s sickle is already distinguishable at the presumptive posterior edge of the blastoderm. At these stages the hypoblast can be separated mechanically (Fig. 1a, b) in both area pellucida and opaca. The quail lower germ layer was associated with the chick epiblast or inversely (Fig. 5) and cultured with the epiblast side down for 24–50 h according to the culture technique devised by New (1955). After *in vitro* culture of the recombinant embryos, the whole blastoderms were fixed in Zenker’s fluid between the 6- and 21-somite stages (Fig. 2). They were cut in 5 μm transverse or longitudinal serial sections subsequently treated by the Feulgen–Rossenbeck staining procedure (1924) by means of which quail and chick cells can be distinguished (Figs. 3 and 4). The extent of the area deriving from the combined lower germ layer was recorded through measurements of the surface occupied by the labelled hypoblast cells, identified by the type of their nucleus (quail or chick according to the combination). The results were reported on a schema for each embryo studied (Fig. 5).

In another series of experiments, heterospecific combinations were carried out at stages 4, 5 and 6 of Vakaët (12–18 h of incubation) corresponding to the formation of the primitive streak. At these stages the lower layer was removed only from the *area pellucida* (Fig. 5).

2. **In vitro associations between chick and quail germ layers at stages 5–7 of H. and H. followed by in vivo culture**

Pieces of the area pellucida from quail and chick embryos at stages 5–7 of H. and H. (stage 5 of H. and H. corresponds to stage 8 of Vakaët) were dissociated, by treatment with 0.1% solution of trypsin in Mg²⁺-Ca²⁺-free Tyrode solution, into ectoderm and endoderm + mesoderm (Fig. 6). Interspecific associations of the ectoderm (involving part of the presumptive lateral plate) with endoderm + mesoderm were made by cultivating the combined tissues for 12 h on a semi-solid culture medium (Wolff & Haffen, 1952). Afterwards, the explant was grafted into the somatopleure of 3-day-old chick hosts for 14 days in order to allow complete histogenesis. Some of the grafts were fixed in Zenker’s fluid and cut in 5/μm serial sections which were stained according to the Feulgen–Rossenbeck’s procedure in order to recognize the respective localization of chick and quail cells. The other grafts were treated by the formol-induced fluorescence technique (FIF) (Falck, 1962) to identify the enterochromaffin cells which are characterized by a significant content of serotonin from 14 days of incubation in chick embryos (Enemar, Falck & Håkanson, 1965) The tissues were quenched
Fig. 1. (a) Cross-section of a chick blastoderm at stage 2 of Vakaët. E, epiblast; H, hypoblast. (b) Epiblast after removal of the hypoblast by mechanical dissociation. Haematoxylin eosin staining.

Fig. 2. Chimaeric blastoderm resulting from the association at stage 2 of Vakaët of the epiblast of a quail embryo with the hypoblast of a chick embryo. Duration of the in vitro culture: 2 days. The embryo was fixed at 12-somite stage.

Fig. 3. Cells of the area pellucida of a chick embryo at stage 2 of Vakaët. Feulgen–Rossenbeck staining.

Fig. 4. Quail cells observed in the similar region at the same stage. Large heterochromatic condensation in the nucleus. Feulgen–Rossenbeck staining.
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Stages of Vakaët

Fig. 5. Diagram showing the various combinations carried out between chick and quail germ layers at stages 2–6 of Vakaët. At stages 2 and 3 the hypoblast is removed from the area pellucida and opaca in quail (BLQ) and chick (BLC) blastoderms. Quail hypoblast (HQ) is associated with chick epiblast (EC) and inversely quail epiblast (EQ) with chick hypoblast (HC). At stages 4, 5 and 6 the endoblast is removed only from the area pellucida. Associations are formed between quail ectomesoblast (EMQ) and chick endoblast (ENC) and inversely between chick ectomesoblast (EMC) and quail endoblast (ENQ). In the right column, the chimaeric embryos are represented at time of fixation. When the combinations are performed at stages 2 and 3 of Vakaët, the hypoblast cells are found in the anterolateral area of the extra-embryonic endoderm (hatched lines). In the embryos of the 3rd experimental series, the grafted lower germ layer was found taking part in the embryonic endoderm.

in melting isopentane cooled in liquid nitrogen. They were freeze-dried for 18 h at –40 °C. Tissues were then exposed to formaldehyde vapour (equilibrated to a humidity of 70%) at 80 °C for 2 h and directly embedded in Epon araldite in vacuo. Serial sections were cut at 5 μm and examined by fluorescence microscopy. After observation in u.v. light, sections were treated as follows: (1) removal of embedding resin according to Mayor, Hampton & Rosario (1961); (2) postfixation in Zenker’s fluid; (3) staining by the Feulgen reaction.

Comparison of the fluorescent and Feulgen photographs of the sections makes it possible to establish whether the fluorescent enterochromaffin cells belong to quail or chick species.
Fig. 6. At stage 5 of H. and H., two different areas of the blastoderm were isolated: A and B, in which the primitive streak was excluded. Ectoderm and endoderm + mesoderm were separated by trypsinization and heterospecifically combined between quail and chick embryos.

A silver impregnation technique after Bouin’s fixation (Ungewitter, 1951) was also used in some cases to demonstrate enterochromaffin and enteric ganglion cells in the explants.

RESULTS

1. In vitro culture of quail-chick chimaeric blastoderms

Out of the 28 embryos resulting from the interspecific association of hypoblast and epiblast of quail and chick embryos at stages 2 and 3 of Vakaët, 27 developed normally and were fixed at 12-somite stage. In all cases the embryonic endoderm of the pharynx and area pellucida was found to be derived from the epiblast of the original association, i.e. it had the same nuclear type as the ectodermal and mesodermal derivatives of the embryo.

The area opaca was divided into two parts with respect to the nuclear type of the endodermal cells: an anterolateral region deriving from the hypoblast and a posterior one, in which the nuclei were of the epiblast type, as in the embryonic endoderm (Figs. 5, 7, 8, 9, 10).

Figures 7-10

Fig. 7. Transverse section through an embryo similar to that represented in Fig. 2. Feulgen–Rossenbeck staining. Details are represented in the following figures.

Fig. 8. Higher magnification of the framed area of Fig. 7. Ectoderm and mesoderm are of quail type; endoderm is of chick type.

Fig. 9. In the same embryo the margin of the blastoderm at the transverse level of the tenth somite shows the extension of the ectoderm and endoderm.

Fig. 10. The pharyngeal endoderm is made up of quail cells, that are derived from the primitive epiblast.
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The border-line of the anterolateral hypoblastic area corresponded in most cases to the frontier between the area pellucida and opaca, i.e. to the junctional area of Vakaët (1970).

Fifteen embryos were operated at stages 4, 5 and 6 of Vakaët (five embryos for each stage) and were fixed at 12- to 21-somite stages. In this series, the exchange of endoblast between chick and quail was restricted to the area pellucida (Fig. 5). The junction between the extra-embryonic endoblast and the germ layer grafted in the area pellucida was completed after a few hours in culture. At the end of the experiment, the embryonic endoderm was found to be mostly of the type of the grafted endoblast (Fig. 5). A significant number of cells derived from the upper germ layer participated however in pharyngeal endoderm formation. They were distributed in large areas located either dorsally, ventrally or laterally in the embryos operated at stages 4 and 5 of Vakaët.

Among five cases where the operation was carried out at stage 6, a few ectomesoblast-type cells were found in the pharynx of only one (Figs. 11, 12). The distribution of ectomesoblast-type cells of the embryos observed is represented on Fig. 13.

2. Interspecific association of ectoderm and endoderm + mesoderm at stages 5–7 of H. and H.

In the explants cultured first in vitro (12 h) and then in vivo (14 days) various tissues and organs developed. Normal histogenesis occurred but the various tissues were anarchically organized in the grafts. Intestinal structures were, however, readily recognizable. The intestinal cells (epithelium, connective and muscular tissue) arose from the endomesoderm of the graft but the enteric ganglia were always of ectoblast type (Fig. 14). In none of the explants observed in serial sections were cells of ectoblast type found in the endodermal epithelium. The latter however contained enterochromaffin cells characterized by their content of fluorogenic monoamines and their affinity for silver salts. The nucleus of the enterochromaffin cells was always of the same type as the other endodermal cells.

CONCLUSION AND DISCUSSION

By time-lapse cinematography, Vakaët (1970) recognized three successive waves of cell movements in the formation of the lower layer of bird blastoderm. From the entophyll (according to the nomenclature of Celestino da Costa (1948)) which is already formed in the young (stage 1) germ of the chick, the first movement is a concentric ingrowth which is responsible for the formation of the so called junctional endoblast situated at the margin of the areas opaca and pellucida. The second wave of cells originates, according to Vakaët, from the posterior border of the area pellucida, at the level of the Koller’s sickle. The ‘sickle endoblast’ progressively extends towards the anterior blastoderm lip, while the very primitive entophyll is pushed cranially. The sickle endoblast is supposed to be derived from multi-invagination of cells from the entophyll.
Fig. 11. Cross-section through a 20-somite chimaeric embryo resulting from the association at stage 4 of Vakaët of quail ectomesoblast and chick endoblast. The pharyngeal endoderm is made up of quail cells originating from the ectomesoblast (Q) and chick cells from the endoblast (C). NC, notochord. Feulgen–Rossenbeck staining.

Fig. 12. Transverse section through an embryo operated at stage 6 of Vakaët and fixed at 15-somite stage. Quail ectomesoblast was associated with chick endoblast. The whole embryonic endoderm is composed of chick cells (C). Q, quail cells in the mesoderm. Feulgen–Rossenbeck staining.
Stages of operation (Vakaët) | Localization of 'ectomesoblast-type' cells (■) in the pharynx at 12-21 somite stages
---|---
4 | 2 embryos | 2 embryos | 1 embryo
5 | 3 embryos | 1 embryo | 1 embryo
6 | 4 embryos | 1 embryo

Fig. 13. Diagram summarizing the results of the experiment represented in Fig. 5, in which ectomesoblast and endoblast were exchanged between quail and chick embryos at stages 4–6 of Vakaët. The pharyngeal endoderm is composed of a mixture of cells of ectomesoblast and endoblast types in all the cases when the combination was performed at stages 4 and 5 and in one out of five cases when the combination was done at stage 6.

Lastly, the lower germ layer is completed by invagination of cells from the ectophyll through the primitive streak. This 'definitive endoderm' (Vakaët, 1970) extends from stage 4 of Vakaët concentrically around Hensen's node and by the end of stage 6 it has reached its most anterior extension.

From stages 6–8 of Vakaët, the endoblast forming activity is no longer concentrated in Hensen's node but extends through the whole primitive streak. At stage 8 invagination of endoblast could be no longer demonstrated either by Vakaët (1970) or by authors using other methods (Gallera & Nicolet, 1969; Nicolet, 1965, 1967, 1970; Rosenquist, 1966).

Our experiments based on chimaeric quail-chick embryos confirm that the lower germ layer of the early bird blastoderm (at stage 1–4 of Vakaët) does not participate in embryonic endoderm formation. They show that the hypoblast migrates cranially and forms the extra-embryonic endoderm of the anterolateral regions of the blastoderm. Moreover, due to the stability of the nuclear marker used in this study, the limits of the endodermal area derived from the primitive hypoblast can be precisely defined. The formation of the embryonic endoderm by migration of cells from the ectomesoblast was also observed. The most active
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Fig. 14. Intestinal structures which developed in the explants composed of chick endomesoderm with ectoblast of quail. The association of the two germ layers was done at stage 6 of H. and H. and the explant was cultured for 14 days. Quail cells originating from the neurectoderm have migrated into the gut structures and given rise to the enteric ganglia (EG), but no quail cells are seen in the gut epithelium. Feulgen–Rossenbeck staining.

phase of endodermal cell migration from the upper germ layer takes place at stages 4 and 5 of Vakaët, but migration is still in progress at stage 6. The cells which migrate during this late endoderm formation period are regularly found in the foregut.

The second experimental series in which endoderm + mesoderm and ectoderm were exchanged between quail and chick embryos, indicates that no migration of cells from the ectoderm into the endoderm occurs during stages 5–7 of H. and H., i.e. before the formation of the neural crest. One must therefore exclude a possible neurectodermal origin for the endocrine cells of the gut epithelium.

These results extend those previously reported (Le Douarin & Teillet, 1974) concerning grafts of fragments of neural primordium between quail and chick embryos at all levels of the neural axis caudal to the mesencephalon. In such experiments, the migration of the grafted neural crest cells into Auerbach’s and Meissner’s plexuses of the host enteric ganglia was demonstrated; but in no case, did cells of crest origin migrate into the endodermal epithelium.

We have shown in a series of previous studies (Le Douarin & Le Lièvre, 1970;
Le Douarin et al. 1974; Polak et al. 1974; Le Douarin, 1974) that the calcitonin-producing cells, which develop in contact with the ultimobranchial endoderm, originate from the rhombencephalic neural crest. Similar conclusions were drawn concerning the type I cells of the carotid body and the fluorogenic amine-containing cells of the wall of the large arterial trunk originating from the heart (Le Douarin, Le Lièvre & Fontaine, 1972; Pearse et al. 1973; Le Lièvre & Le Douarin, 1973, 1975). Those cells, with the adrenomedullary paraganglia, the enterochromaffin and endocrine cells of the gut epithelium and also the pancreatic islet cells, belong to the APUD series of Pearse.

From our experimental results we cannot confirm the very attractive hypothesis of a common embryological origin for all the APUD cells from the neural crest. We can however conclude that cells of the early neural ectoderm do not contribute to endoderm formation before the stage at which the neural crest is individualized.

Theoretically, only one possibility remains for a cell line originating from the presumptive ectoderm to participate in the formation of the endodermal germ layer. As previously shown, the endoderm is formed by migration of cells from the upper germ layer through Hensen's node and the primitive streak. No experiments available at the present time exclude the possibility that cells of the ectoblastic area, already determined as ectodermal cells, participate in endoderm formation even if most of them remain in the superficial germ layer. It must be underscored however that although there are no experimental proofs to rule out this hypothesis, no argument supports it either.

Of course, it would be attractive if a common origin of polypeptide secreting cells from one germ layer accounted for some common cytochemical, structural and biochemical characteristics. Unfortunately no embryological information makes it possible to attribute beyond doubt a common ancestor to the neuroectodermal endocrine cells.

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