Differentiation of allantoic endoderm implanted into the presumptive digestive area in avian embryos. A study with organ-specific antigens

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SUMMARY

Quail allantoic endoderm was implanted into the presumptive digestive-tract area of chick embryos, and the differentiation of the endoderm was examined morphologically and immunocytochemically with antisera against pepsinogens and sucrase. The allantoic endoderm was incorporated into the host digestive organs. It often became continuous with the host endoderm and formed a chimaeric digestive-tract epithelium. It differentiated morphologically into the epithelium of the digestive organ into which it was incorporated, showing the morphological inductive ability *in situ* of the digestive-tract mesenchyme against the allantoic endoderm. However, the allantoic endoderm did not produce pepsinogens even when it was incorporated into the host proventricular mesenchyme and formed well-developed proventricular glands. This result indicates that the heterotypic morphogenesis of the allantoic endoderm is not necessarily accompanied by the heterotypic cytodifferentiation. In contrast, the anti-sucrase antiserum-reactive cells often differentiated in the allantoic endoderm incorporated into not only the intestine but also other organs. This confirmed our previous observation that the allantoic endoderm has a tendency to differentiate into the intestinal epithelium in the heterologous environment.

INTRODUCTION

It is well established that tissue interactions are prerequisite for the morphogenesis and cytodifferentiation of many developing tissues and organs (Saxén *et al.* 1976; Wessells, 1977; Sawyer & Fallon, 1983). The examples of instructive tissue interactions leading to the heterotypic morphogenesis of effector tissues according to the origin of inductor tissues are numerous. Although it is quite conceivable that specific morphological changes involve the expression of specific genetic informations, it is difficult at the moment to analyse the causal relationships between them. However, several examples of tissue interactions which bring about the heterotypic cytochemical differentiation of effector tissues have been described. Among them are: (a) lens crystallin induction in trunk epidermis by optic vesicle in the avian embryo (Karikinen-Jääskeläinen, 1978), (b) feather or scale keratin formation in epidermis by specific induction of dermis obtained from the feather- or scale-forming region (Dhouailly, Rogers & Sengel, 1978), (c) type II collagen synthesis in mouse tooth mesenchyme cultivated

combined with avian limb-bud epidermis (Hata & Slavkin, 1978), (d) enamel synthesis in chick pharyngeal endoderm recombined with mouse molar mesenchyme (Koller & Fisher, 1980), (e) prostatic differentiation of the epithelium of adult rat urinary bladder under the influence of urogenital-sinus mesenchyme (Cunha et al. 1983; Neubauer et al. 1983).

Mesenchyme of digestive organs in avian embryos has been shown to exert region-specific inductive influence on allantoic and yolk-sac endoderm and digestive-tract epithelium (Mizuno & Yasugi, 1973; Yasugi & Mizuno, 1974, 1978; Yasugi, 1976a, 1976b, 1979; Gumpel-Pinot, Yasugi & Mizuno, 1978; Mizuno & Masui, 1982; Haffen, Kedinger, Simon-Assmann & Lacroix, 1982). The last-mentioned authors showed that small-intestinal mesenchyme induced sucrase and maltase activities in recombined gizzard endoderm. This is another example of tissue interactions leading to heterotypic epithelial cytodifferentiation.

All the experiments mentioned above were carried out with tissues cultivated by various in vitro and grafting methods. It is generally assumed that in vitro or grafting experiments reflect the normal inductive ability of the inductor tissues in situ. However, there have been no confirmative experiments to prove this. It is important to ascertain the inductive ability in situ in order to analyse the process of tissue interactions in the normal course of development.

The aims of the present study are, firstly to investigate the inductive ability in situ of the mesenchyme of avian digestive organs against the allantoic endoderm; secondly to find whether allantoic endoderm can respond to the inductive ability, if any, of the mesenchyme, specifically by producing functional proteins specific to the digestive-tract epithelia.

Quail allantoic endoderm was implanted into the presumptive digestive-tract area of chick embryos, and the differentiation of the endoderm was examined morphologically and immunocytochemically with antisera against embryonic and adult pepsinogens specific to chick and quail proventricular epithelium (Yasugi & Mizuno, 1981a, 1981b), and against chick small-intestinal sucrase (Matsushita, 1983).

MATERIALS AND METHODS
Tissue isolation and implantation

Allantoic endoderm was obtained from the allantois of 3-day-old Japanese quail (*Coturnix coturnix japonica*) embryos by treatment with collagenase (Worthington Biochemicals Co., Code CLS, 0·03 % in Tyrode's solution, for 30 min at 37 °C). After isolation, the endoderm was washed thoroughly in serum-supplemented Tyrode's solution, then in Tyrode's solution. Proventricular endoderm was also isolated by the same treatment from 5-day-old quail embryos.
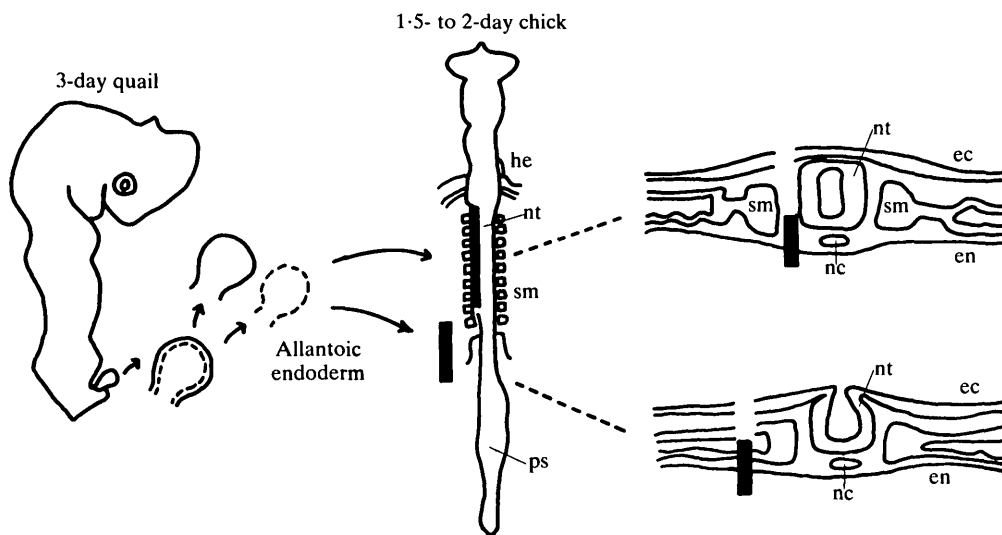
Fig. 1. Diagram showing the mode of implantation of the quail allantoic endoderm into the presumptive digestive tract of the chick embryo (see Methods for further details). he: heart; nt: neural tube; sm: somites; ps: primitive streak; nc: notochord; ec: ectoderm; en: endoderm.

Isolated quail endodermal fragments were implanted into the presumptive digestive area of 1·5- to 2-day-old chick (*Gallus gallus domesticus*) embryos, as shown in Fig. 1, together with a number of carbon particles to identify grafted tissue. To introduce the endoderm into the oesophageal, proventricular, or gizzard mesenchyme, it was necessary to implant it in the area slightly lateral to the neural tube at the level of somite 1 to 7. The grafts implanted more caudally or more laterally were incorporated mostly into the intestinal region of hosts.

The grafts were recovered with the aid of the carbon particles usually 10 days after implantation, but in some cases several days after hatching of the hosts.

Histological and immunocytochemical procedures

Grafts with adjacent host tissues were fixed in Bouin's fluid for PAS-haematoxylin (HX) staining or in ice-cold 95 % ethanol for 4 h for indirect immunofluorescence studies, according to the method of Sainte-Marie (1962). All sections were cut at 5 μm and, in the case of immunofluorescence studies, they were serially mounted onto three slides: one for PAS-HX, one for the detection of pepsinogen and one for sucrase. After observation by immuno-fluorescence microscopy, the same slides were stained with PAS-HX. The FITC-conjugated anti-rabbit IgG serum of goat was purchased from Miles Laboratories Inc. (Indiana, USA).

Pepsinogens specific to the embryonic (EPg) and adult (APg) chick and quail proventriculus (Yasugi, Mizuno & Esumi, 1979; Yasugi & Mizuno, 1981a) were
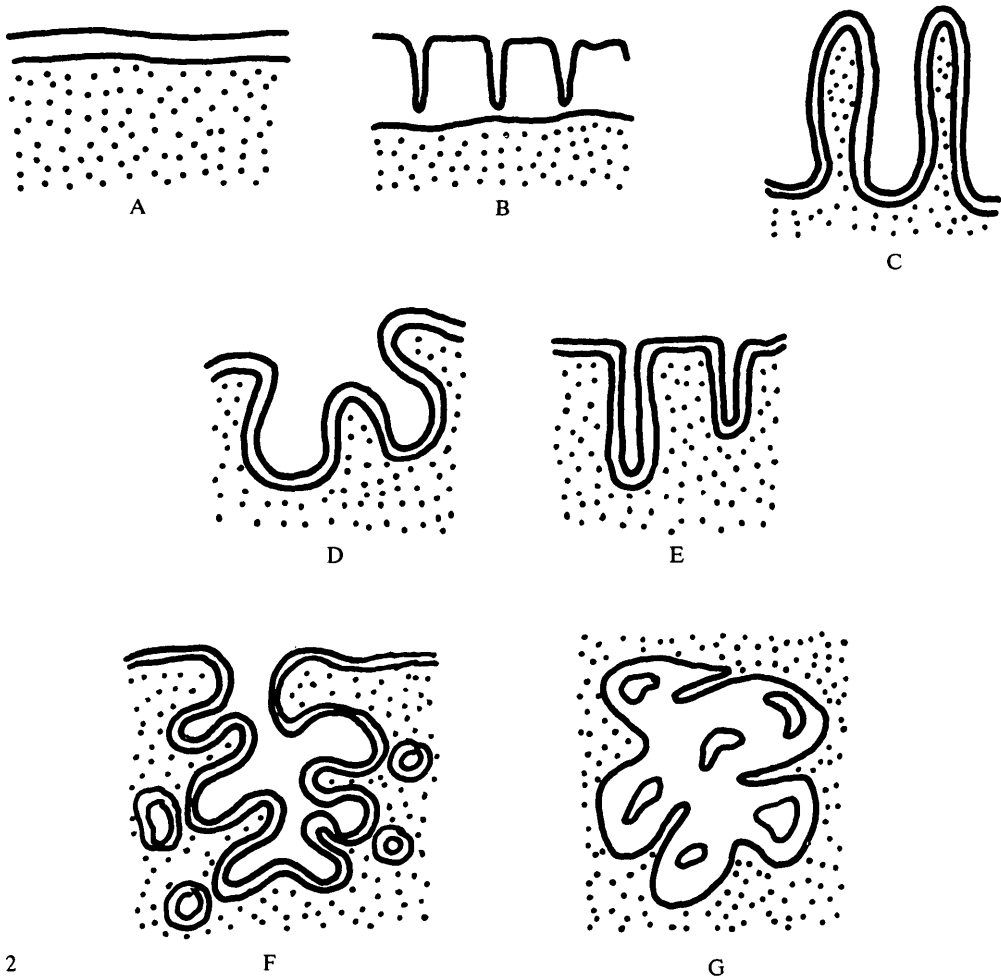
Fig. 2. Schematic drawings showing seven categories of epithelial morphology. Dotted area represents the mesenchyme. (A) Flat epithelium. (B) Epithelium with vertical striations. (C) Villi. (D) Folds. (E) Invaginations. (F) Glands. (G) Cell mass with intra-epithelial lacunae.

Fig. 3. Quail allantoic endoderm incorporated into the oesophagus (10 days' graft). The epithelium between the two lines comprises quail cells. Bouin-fixed, PAS-HX. ×270.

Fig. 4. Quail allantoic endoderm incorporated into the oesophagus (10 days' graft). Note the connective tissue layers induced around the grafted quail epithelium. Ethanol-fixed, PAS-HX. ×124.

Fig. 5. Quail allantoic endoderm incorporated into the oesophagus and recovered after hatching of the host. Mucus-secreting gland cells and goblet cells (arrow) differentiated. Host chick oesophagus with oesophageal glands is seen on the left. l: lumen of the host oesophagus. Ethanol-fixed, PAS-HX. ×70.
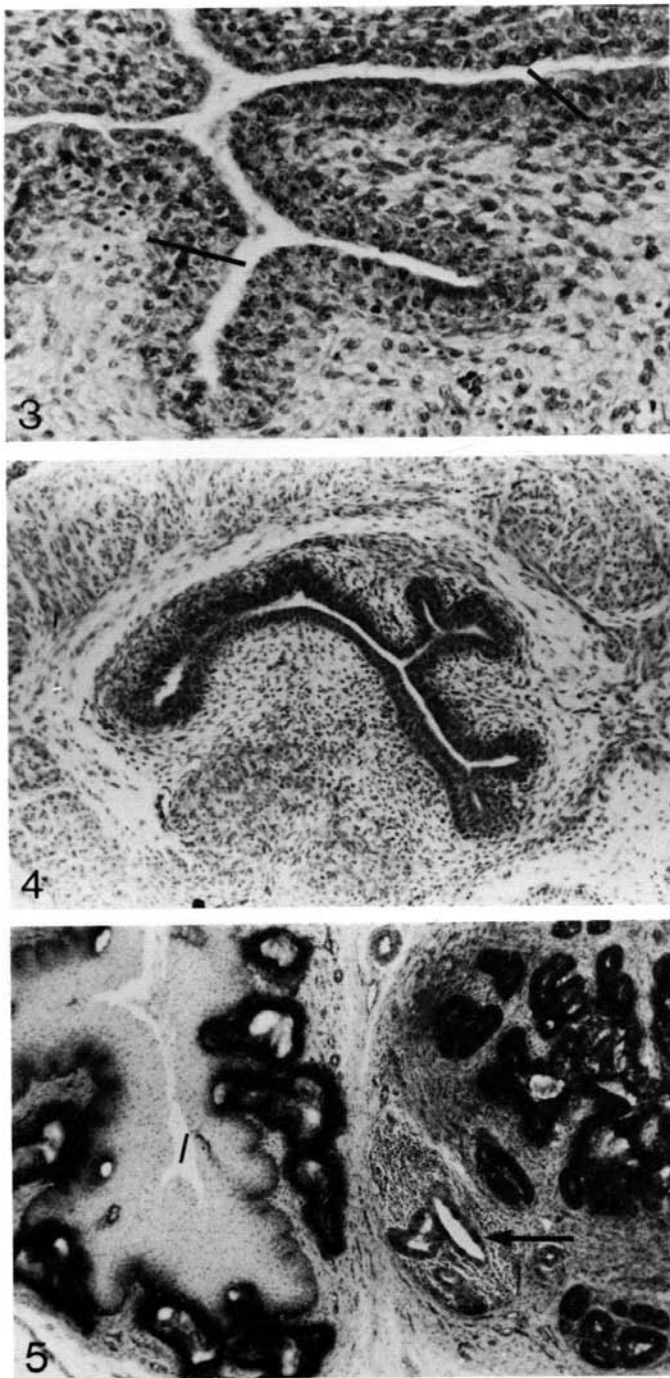
Figs. 3–5

purified as described earlier (Esumi, Yasugi, Mizuno & Fujiki, 1980; Yasugi & Mizuno, 1979, 1981b) and antisera against these pepsinogens were prepared in the rabbits. The antisera were absorbed with the precipitate obtained by centrifugation at 40 000 r.p.m. for 1 h of the homogenate of the chick oesophagus. As was previously reported (Yasugi & Mizuno, 1981b, 1982), these antisera showed no species specificities between chick and quail but showed organ- and developmental-stage specificities; anti-EPg antiserum reacted with gland cells of both chick and quail embryonic proventriculus but not with those of hatched chick or quail. Conversely, anti-APg antiserum stained adult proventricular gland cells of both chick and quail specifically. Tissues other than the proventriculus never showed cross reactivity to those antisera.

The antiserum against purified chick small-intestinal sucrase was kindly donated by Dr Matsushita. This antiserum was shown to react with only the small-intestinal brush border of both the chick and quail (Matsushita, 1983).

Analysis of epithelial differentiation

Only grafts found in the definite mesenchyme of the host digestive tract were analysed. The biological marker of different nuclear morphology between chick and quail (Le Douarin, 1973) was used to distinguish grafted tissue from host tissue. As shown in Table 1, in the case of 10 days' cultivation, the epithelial morphology was classified into seven categories according to the sketches in Fig. 2. When several categories of differentiation were found together, all of them were scored. Frequencies of appearance of goblet cells, of anti-EPg or anti-APg antiserum-positive cells and of anti-sucrase antiserum-positive cells were scored as epithelial cell differentiation. Since the immunofluorescence studies were not carried out on all of the grafts, the number of grafts examined and the number of positive cases are shown in Table 1.

RESULTS

Allantoic endoderm incorporated into the oesophageal mesenchyme

Allantoic endoderm implanted at the level of somites 1–6, especially 2–4, were recovered in the oesophageal mesenchyme. The endoderm formed chimaeric epithelium with host oesophageal epithelium (9 cases out of 22, Fig. 3), or existed in the mesenchyme independent of the host epithelium (Fig. 4). In the majority of the grafts, the endoderm was folded and, as in host oesophageal epithelium, there were two to three layers of cuboidal endodermal cells. Around the quail epithelium connective tissue cells were condensed and a thick muscle layer was induced (Fig. 4). In the allantoic endoderm found in the oesophageal mesenchyme, no goblet cells differentiated, nor were anti-EPg or anti-sucrase antiserum-positive cells observed.

In the grafts recovered from the oesophageal region after hatching of the hosts

Table 1. *Differentiation of the allantoic endoderm incorporated into various digestive-organ mesenchymes and recovered 10 days after implantation*

Host mesenchyme into which grafted endoderm was incorporated	No. of grafts	Epithelial morphology (%)*							Epithelial cell differentiation		
		flat	vertical striations	villi	folds	invagi-nations	glands	cell mass with lacunae	goblet cells (%)	anti-EPg positive cells†	anti-sucrase positive cells†
Oesophagus	22	18	0	0	86	5	5	5	0	0/12	0/10
Proventriculus	19	0	0	0	0	21	74	68	11	1/15	3/10
Gizzard	9	56	67	0	33	0	0	11	0	0/7	3/6
Small intestine	17	29	0	18	53	0	0	0	0	0/7	3/7

* See Fig. 2. for the classification of categories.
† Number of grafts having cells indicated/number of grafts examined.

(two cases), the allantoic endoderm formed glandular structures actively secreting mucus like host oesophageal glands (Fig. 5). Only in the limited area, the grafted endoderm differentiated into pluristratified epithelium. In both grafts, anti-sucrase antiserum-positive cells and goblet cells (Fig. 5) were detected.

Allantoic endoderm incorporated into the proventricular mesenchyme

Allantoic endoderm was often found in the host proventricular mesenchyme when it was implanted at the level of somite 3–5. The endoderm existed in the mesenchyme independent of the host proventricular epithelium (Fig. 6), or it formed chimaeric epithelium with luminal epithelium of the host proventriculus (7 cases out of 19; Fig. 7). In both cases, the stratified columnar or cuboidal epithelium invaginated into the stroma and often formed glandular structures or sometimes a cell mass with intra-epithelial lacunae. Goblet cells were observed in 2 cases out of 19.

In 14 out of 15 grafts examined, no anti-EPg antiserum-positive cells were observed. In some cases the quail allantoic endoderm formed well-developed glands only about 50 μm apart from the host glands, but the quail glands were negative, in sharp contrast to the positive host glands (Figs 7 and 8). Only in one graft, a few anti-EPg antiserum-positive cells appeared in the quail epithelium invaginated into the host mesenchyme.

The anti-sucrase antiserum-positive cells were found in three cases out of ten examined. Clusters of cells were immuno-fluorescent at the apical surface. They were most obvious at the villus-like part of the epithelium, but other parts were also occasionally positive.

Fig. 6. Quail allantoic endoderm (q) incorporated into the proventriculus (10 days' graft). The grafted quail epithelium is independent of the host chick glands (c) and has a well-developed muscular layer (m) around it. l: lumen of the host proventriculus. Ethanol-fixed, PAS-HX. ×80.

Fig. 7. Quail allantoic endoderm (q) incorporated into the proventriculus (10 days' graft). c: glands of the host chick; l: lumen of the host proventriculus. Ethanol-fixed, PAS-HX after immunofluorescence. ×90.

Fig. 8. The same section as in Fig. 7. Immunofluorescence for EPg. Host chick glands are positive, while quail epithelium is negative. The positive materials in the upper right part are secreted substances. ×90.

Fig. 9. Quail allantoic endoderm (q) incorporated into the proventriculus and recovered after hatching of the host. Chick cells (c) differentiated into typical gland cells, while quail cells are mucus-secreting. Ethanol-fixed, PAS-HX after immunofluorescence. ×130.

Fig. 10. The same section as in Fig. 9. Immunofluorescence for APg. Chick cells are positive, while quail cells are negative. ×130.

Fig. 11. Quail allantoic endoderm incorporated into the proventriculus and recovered after hatching of the host. Goblet cells (arrows) are seen. Ethanol-fixed, PAS-HX after immunofluorescence. ×400.

Fig. 12. The same section as in Fig. 11. Immunofluorescence for sucrase. ×400.
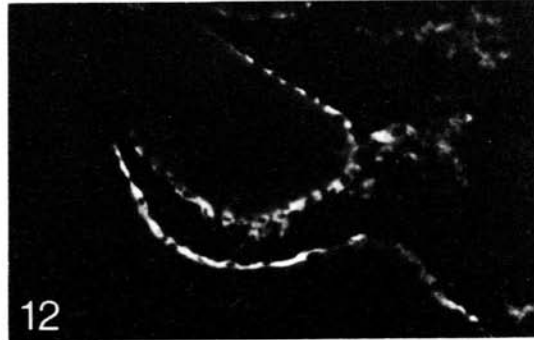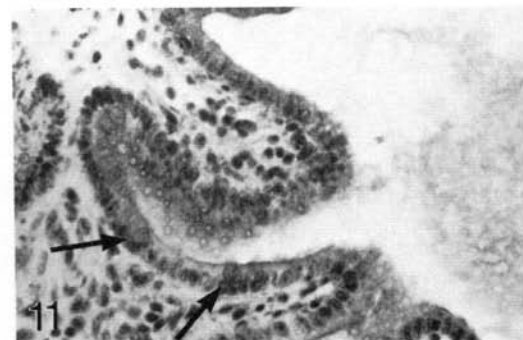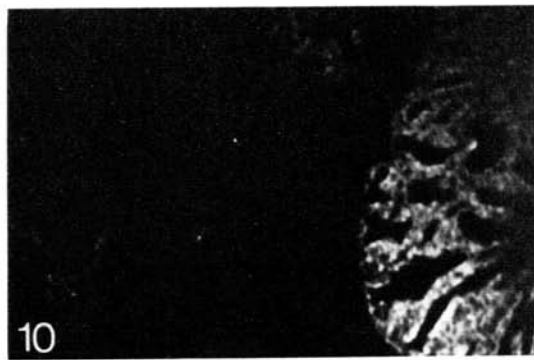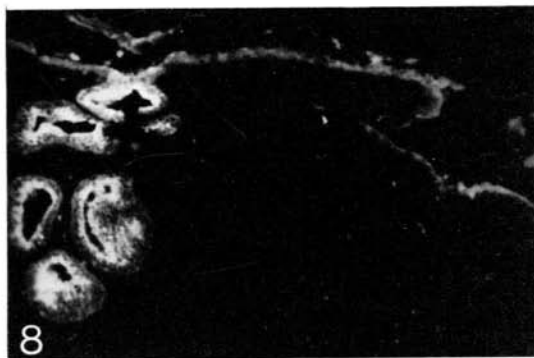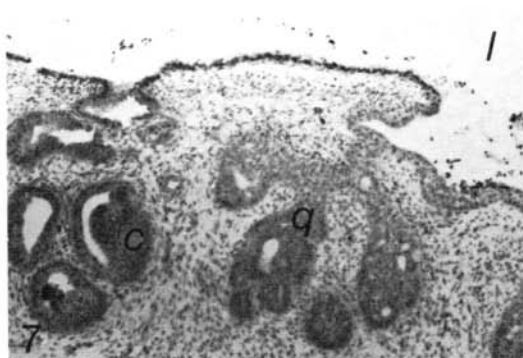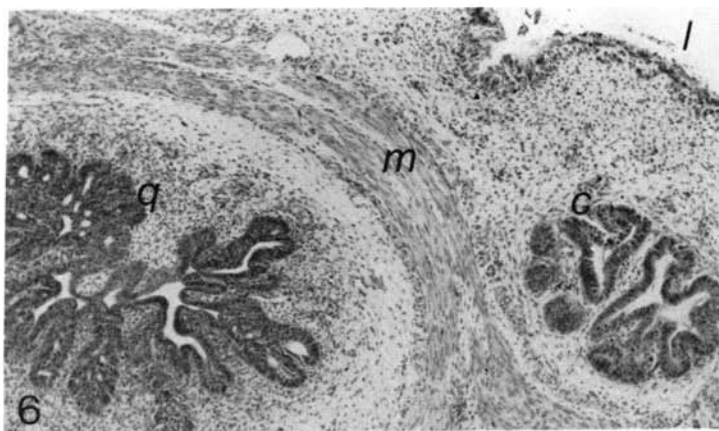
Figs. 6–12

In the hatched chick proventriculus, the explanted quail allantoic endoderm (two cases) continued to form glands (Fig. 9) but the cellular differentiation was quite different from that observed in short-term cultivation. The majority of the quail cells secreted mucus and goblet cells were also observed (Fig. 11). In the implanted quail epithelium, anti-EPg or anti-APg antiserum-positive cells were never detected while host gland cells reacted with anti-APg antiserum (Figs 9 and 10). The free surface of some quail epithelial cells was positive against anti-sucrase antiserum (Figs 11 and 12).
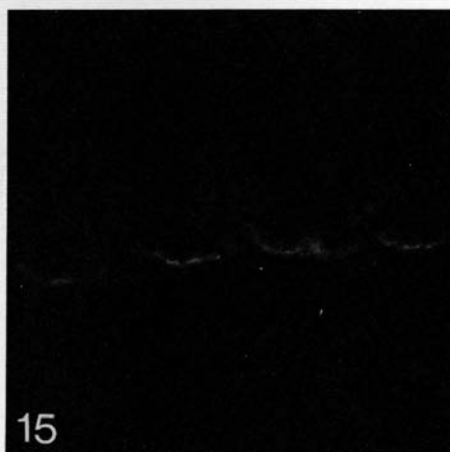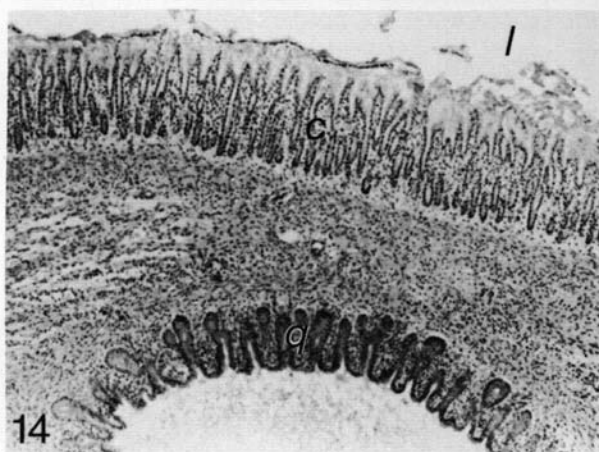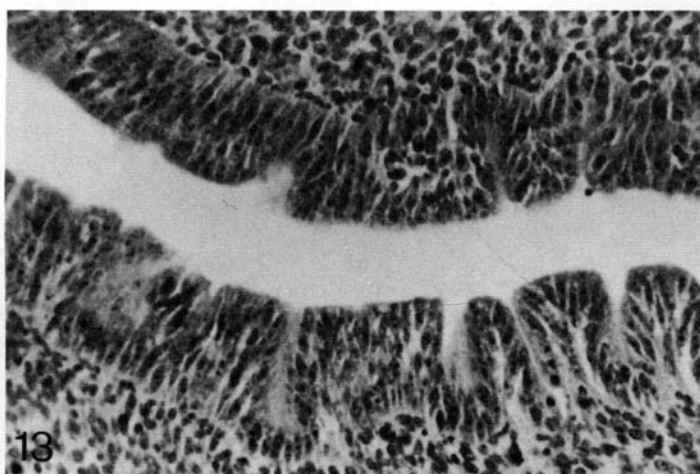

Fig. 13. Quail allantoic endoderm incorporated into the gizzard (10 days' graft). The epithelium is stratified columnar (upper left) and with vertical striations (lower). Ethanol-fixed, PAS-HX. ×270.

Fig. 14. Quail allantoic endoderm (q) incorporated into the gizzard and recovered after hatching of the host. Quail epithelium forms many invaginations, while chick epithelium (c) differentiated into typical gizzard epithelium. 1: lumen of the host gizzard. Ethanol-fixed, PAS-HX after immunofluorescence. ×70.

Fig. 15. The same section as in Fig. 14. Immunofluorescence for sucrase. Free surfaces of the quail epithelial cells are positive. ×390.

Allantoic endoderm incorporated into the gizzard mesenchyme

Allantoic endoderm incorporated into the gizzard mesenchyme was found in grafts made mainly at the level of somite 4–6. The continuation of the host gizzard epithelium and the grafted allantoic endoderm was observed only in one case out of nine, with the grafted epithelium usually situated deep in the host gizzard mesenchyme. The grafted endoderm was flat epithelium or epithelium with vertical striations and comprised stratified columnar or cuboidal cells (Fig. 13). Connective tissue layers of chick cells were induced around the grafts.

Goblet cells and anti-EPg antiserum-positive cells were never observed, while anti-sucrase, antiserum-positive cells differentiated in three cases out of six examined.

After hatching of the hosts, the differentiation of implanted quail allantoic endoderm (two cases) was similar to that in short-term cultivation. It differentiated into a simple columnar epithelium with vertical striations or with glandular invaginations like the host gizzard epithelium (Fig. 14). In both cases abundant goblet cells were observed, and in one graft anti-sucrase, antiserum-positive cells were detected (Fig. 15).

### *Allantoic endoderm incorporated into the small-intestinal mesenchyme*

If the allantoic endoderm was grafted at a level posterior to somite 7 or in the lateral part of the embryo, it was incorporated into the small-intestinal mesenchyme of the host. In 9 cases out of 17, the grafted endoderm was continuous with the host small-intestinal epithelium. In other cases the grafted endoderm often extended lengthwise as an independent small intestine involving the chick mesenchyme around it (Fig. 16). The quail epithelium was simple columnar or simple cuboidal and took the form of a simple tube or a tube with villi or folds. Goblet cells did not differentiate in grafts and in host small intestine during 10 days' cultivation. Anti-sucrase antiserum-positive cells were observed in three grafts out of seven examined (Figs 17 and 18). The small intestine of the host occasionally expressed sucrase at this developmental stage (12-day-old), but there was no correlation between positive reactions against anti-sucrase antiserum of hosts and those of grafts (Fig. 18).

Proventricular endoderm incorporated into the proventricular mesenchyme

As a positive control, the proventricular endoderm of 5-day-old quail embryos was implanted into the presumptive proventricular area of the chick embryos. The grafted endoderm incorporated into the host proventriculus often showed continuity with host proventricular epithelium and formed well-developed glands (Fig. 19). Both gland cells and secreted materials from the grafted epithelium were anti-EPg antiserum-positive just as the host proventriculus (Fig. 20).

DISCUSSION

When discussing the morphogenesis and cytodifferentiation of the digestive-tract epithelium in avian embryos, it has been suggested that it is necessary to consider at least two factors, that is, the self-differentiation potency of the epithelium and the inductive action of the mesenchyme (Mizuno, 1975). The
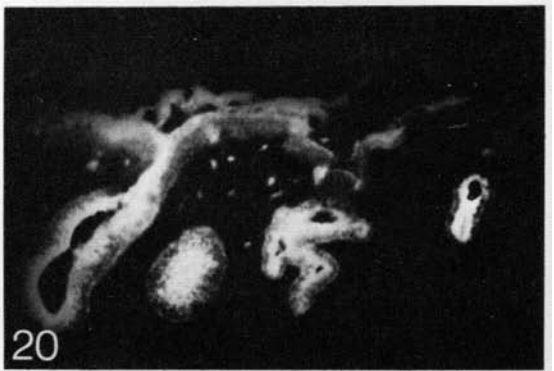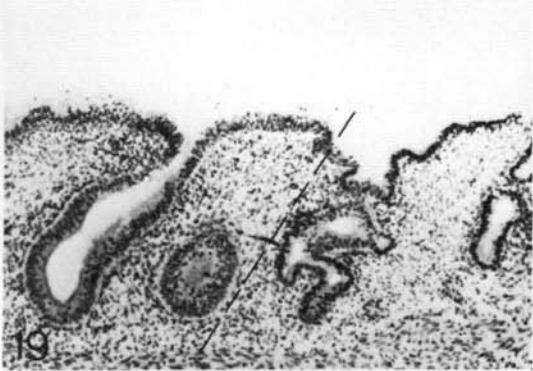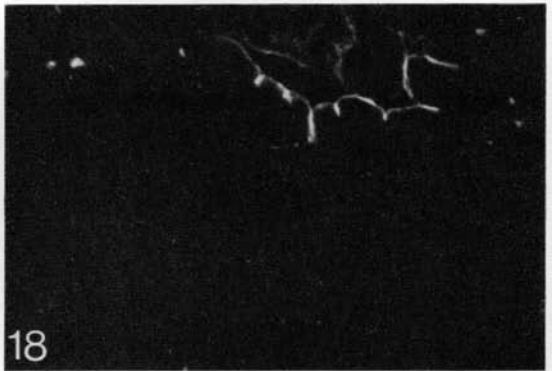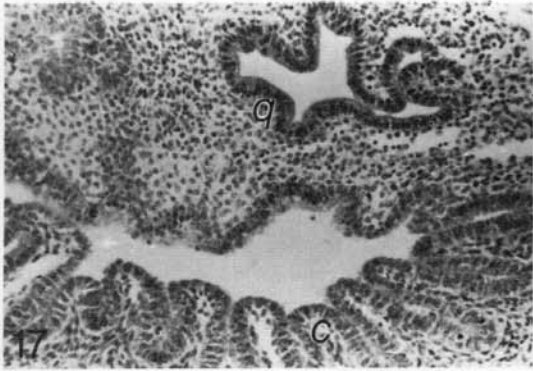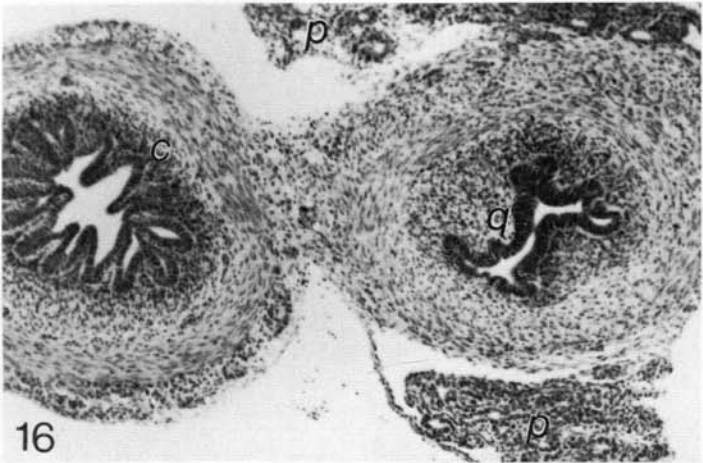
epithelium can self-differentiate to a considerable extent *in vitro* according to its own developmental fate in the absence of the mesenchyme (Mizuno & Sumiya, 1974, 1977; Sumiya & Mizuno, 1974, 1976; Sumiya, 1976), and this potency of the epithelium was confirmed in very young endoderm with electron microscopical criteria (Mizuno & Ishizuya, 1982; Ishizuya-Oka, 1983). The digestive-tract mesenchyme can exert inductive influence on the allantoic endoderm (Mizuno & Yasugi, 1973; Yasugi & Mizuno, 1978; Yasugi, 1976a, 1976b, 1979; Gumpel-Pinot *et al.* 1978), on the digestive-tract endoderm (Gumpel-Pinot *et al.* 1978; Yasugi & Mizuno, 1978; Haffen *et al.* 1982, 1983), on the yolk-sac endoderm (Masui, 1981; Mizuno & Masui, 1983), and even on the epidermis (McLoughlin, 1961). The inductive action of the mesenchyme has been mainly investigated morphologically, but Haffen *et al.* (1982) showed that the small-intestinal mesenchyme induces sucrase and maltase activities in the recombined gizzard epithelium. These experiments were carried out with tissues removed from the embryos.

In the present study, designed to investigate the morphological and cytochemical differentiation of effector tissues *in vivo* under the influence of digestive-tract mesenchyme *in situ*, the quail allantoic endoderm was implanted into the presumptive digestive-tract area of chick embryos and the morphological differentiation and the appearance of organ-specific antigens such as pepsinogens and sucrase were examined.

The results revealed first that the mesenchyme has region-specific inductive ability *in situ*: the allantoic endoderm often showed epithelial morphology and cellular arrangement similar to those of the digestive organs into which it was incorporated.

The second and the most important outcome of the present study was the fact that the allantoic endoderm, when incorporated into the proventricular mesenchyme and cultivated for 10 days or till even after the hatching of the hosts, was found to have differentiated into epithelium negative to the anti-EPg or anti-APg antiserum, except in one positive case. Even in the latter case the positive

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Fig. 16. Quail allantoic endoderm (q) incorporated into the small intestine (10 days' graft). Quail epithelium is independent of host chick epithelium (c) and is surrounded by chick connective tissue layers. P: pancreas of the host. Ethanol-fixed, PAS-HX. ×75.

Fig. 17. Quail allantoic endoderm (q) incorporated into the small intestine (10 days' graft). c: chick small intestine. Ethanol-fixed, PAS-HX. ×150.

Fig. 18. The same graft as in Fig. 17. Immunofluorescence for sucrase. The quail epithelium is positive, while chick epithelium is virtually negative. ×150.

Fig. 19. Quail proventricular endoderm incorporated into the proventriculus (10 days' graft). The surface and gland epithelium to the right of the broken line comprises quail cells. Ethanol-fixed, PAS-HX after immunofluorescence. ×85.

Fig. 20. The same section as in Fig. 19. Immunofluorescence for EPg. Both quail and chick gland cells are positive. ×85.

cells were extremely few and it is difficult to conclude that a fully instructive induction against the allantoic endoderm had occurred. Rather, the fact that in the majority of cases, when the well-developed glands were derived from the grafted allantoic endoderm and were located in close proximity to the host glands, the EPg- or APg-producing cells did not differentiate, indicates that it is difficult for the proventricular mesenchyme to induce the EPg or APg in the grafted allantoic endoderm.

The failure of the detection of pepsinogens in the grafted allantoic endoderm may be due to one of the following possibilities. (a) The inductive influence of the proventricular mesenchyme cannot act on the expression of the genes coding for pepsinogens. (b) Transcription of the pepsinogen mRNA occurred but the post-transcriptional or translational processes cannot proceed in the allantoic endodermal cells. (c) The sensitivity of the anti-EPg and anti-APg antisera is too low. (d) The present experimental conditions are not favourable for the production of pepsinogens in the grafted tissues. (c) and (d) do not seem to be the case because (i) with the antiserum employed in the present study EPg can be detected in the normal proventriculus of 9-day-old chick embryos (Yasugi & Mizuno, 1982), whose peptic activity was very low, and (ii) the quail proventricular endoderm could produce EPg when it was incorporated into the chick proventricular mesenchyme under the same experimental conditions. The possibility of (b) shall be studied further by extracting the mRNAs of pepsinogens and translating them *in vitro*.

In the previous paper (Yasugi, 1976a), acid protease activity was detected in the allantoic endoderm recombined with the proventricular mesenchyme and cultivated on the chorio-allantoic membrane. In the light of the present study, whether or not this activity was due to EPg is a very important and interesting problem and is now being investigated by biochemical and enzymological methods.

At any rate, the present results strongly support the idea that the heterotypic morphogenesis of an epithelial tissue induced by a heterologous mesenchyme is not necessarily accompanied by heterotypic cytochemical differentiation, and these two events are separable (Yasugi & Mizuno, 1974; Sakakura, Nishizuka & Dawe, 1976; Haffen *et al.* 1982).

Sucrase, an enzyme specific to the small-intestinal epithelial cells, was often expressed in the allantoic endoderm when it was implanted into the digestive-tract mesenchymes. This confirms the observations made earlier that the allantoic endoderm has a tendency to differentiate into intestinal epithelium under heterologous conditions (Fell, 1954; Yasugi, 1979). Masui (1981) and Mizuno & Masui (1983) reported that yolk-sac endoderm also has a tendency to differentiate into intestinal epithelium under the influence of digestive tract mesenchymes.

Another interesting point worth mentioning is that connective tissue and muscle layers were often formed around the implanted allantoic endoderm

independently of the host connective tissue layers. This means that either the allantoic endoderm, once committed by the mesenchyme, then became able to organize the host mesenchymal cells into connective tissue and muscle layers, or the allantoic endoderm only stimulated the autonomous tendency of the mesenchyme for condensation and differentiation into connective tissue and muscle layers. Le Douarin and Bussonnet (1966) and Le Douarin, Bussonnet & Chaumont (1968) demonstrated that pharyngeal endoderm, when implanted into the somatopleure, induced the mesenchyme to constitute connective tissue layers, and regarded this phenomenon as an example of induction by the epithelium.

The frequency of goblet cell differentiation in the allantoic endoderm implanted into the presumptive digestive-tract area and recovered after 10 days was much lower than in the allantoic endoderm recombined with digestive-tract mesenchyme and cultivated *in vitro* (Mizuno & Yasugi, 1973; Yasugi & Mizuno, 1974) or on the chorio-allantoic membrane (Yasugi, 1976a, 1976b, 1979). With *in vitro* cultivation, goblet cells appeared after 2 weeks. In the case of cultivation on the chorio-allantoic membrane, the age of the host at the end of their cultivation was 19 to 20 days (Yasugi, 1976a, 1976b, 1979) whereas in the present study the age of the host at the end of cultivation was 12 days, when the goblet cells are very scarce in the normal small intestine. These facts, together with the present observation that the allantoic endoderm differentiated goblet cells when it was recovered after hatching of the hosts, suggest that the appearance of goblet cells in the allantoic endoderm is closely related to the aging processes regulated by some intrinsic or extrinsic environmental factors.

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