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Syndactyly induced by Janus Green B in the embryonic chick leg bud: a reexamination

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SUMMARY

In an attempt to clarify the mechanism of production of the syndactyly induced by Janus Green B (JGB) we have studied the morphology and structural modifications of the chick embryo leg bud after JGB administration by means of (1) neutral red vital staining, (2) whole-mount cartilage staining and (3) light microscopy and transmission and scanning electron microscopy. The results show that the well-known inhibition of interdigital cell death is accompanied by a precocious alteration of the epithelial tissue and especially of the epithelial–mesenchymal interface. 24 h after JGB administration the cells of the AER reduce the number of junctions and the basal ectodermal cells are detached into the mesenchymal tissue in zones in which the basal lamina undergoes disruption. In addition the interdigital mesenchymal cells diverted from the dying program are able to undergo a rapid differentiation into cartilage.

It is proposed that the mechanism of production of JGB-induced syndactyly might be due to an alteration of the normal epithelial–mesenchymal interactions rather than to a direct inhibitory effect of the JGB on the dying program.

INTRODUCTION

The development of the digits of most vertebrates takes place by their detachment from an initial hand or foot plate. Concomitant with the digit-detachment process, prominent necrotic zones are observed in the regressing interdigital spaces of birds, reptiles and mammals (Ballard & Holt, 1968; Fallon & Cameron, 1977; Hinchliffe, 1982; Pautou, 1974; 1975; Saunders & Fallon, 1967; Saunders, Gasseling & Saunders, 1962). Animals displaying webbed digits show a conscipuous reduction of the extension and intensity of the interdigital necrotic zones (INZ) (Fallon & Cameron, 1977; Hinchliffe, 1982; Pautou, 1974; Saunders & Fallon, 1967). Furthermore, INZs are absent in chick mutants showing syndactyly (Hinchliffe & Thorogood, 1974). On the basis of all these results, the formation of free digits is usually considered as a classic case of controlled cell death shaping morphogenesis. There is, however, increasing evidence suggesting that the formation of the free digits may be a more complex mechanism involving changes in all the interdigital tissue

components. In vertebrates with free digits the ectodermal cells which cover the regressing interdigital spaces undergo extensive changes in shape (Hurle & Colvee, 1982) and structure (Kelley, 1973), while the changes are less pronounced or absent in the webbed foot of the developing duck (Hurle & Colvee, 1982). Furthermore, the AER, a well-known differentiated zone of the limb ectoderm which plays a crucial role in the proximodistal growth of the limb (Zwilling, 1968) regresses in the interdigital spaces concomitantly with the onset of INZ (Hurle & Fernandez-Teran, 1983). Finally, in the later stages of digit detachment, the basal ectodermal lamina ruptures and large amounts of collagen appear progressively deposited at the epitheliomesenchymal interface of the regressing interdigital spaces (Hurle & Fernandez-Teran, 1983). Again, these changes are less pronounced in the developing webbed foot of the duck (Hurle & Fernandez-Teran, 1984). Thus, it appears reasonable to assume that not only cell death but also changes in the ectodermal tissue and extracellular matrix interface are involved in the disappearance of the interdigital tissue; however, possible causal relationship between these processes and their relative contribution to the disappearance of the tissue remains to be clarified.

The administration of Janus Green B (JGB) to chick embryos at an appropriate dosage and time, provides a valuable approach for experimental induction of syndactyly (Menkes & Deleanu, 1964; Pautou, 1976). Since JGB appears to reduce or inhibit INZ as revealed by vital staining (Menkes & Deleanu, 1964; Deleanu, 1965), this approach has been currently described as an experimental demonstration of the decisive role of cell death in the formation of the digits. There are however several reports showing that the ectodermal tissue, including the AER, undergoes significant structural changes after JGB administration (Pautou, 1978; Pautou & Kieny, 1971; Fallon, 1972a; and unpublished work of Marsh quoted by Hinchliffe, 1981). These changes have been related to the production of hypophalangy which usually accompanies syndactyly, but their possible involvement in the genesis of the syndactyly has gained interest in light of the recent studies on the role of the ectodermal tissue in the formation of free digits (Hurle & Colvee, 1982; Hurle & Fernandez-Teran, 1983; 1984; Kelley, 1973).

In the present work we have surveyed the morphology and structural modifications of the chicken of the chick leg after JGB administration in order to obtain new information on the role of the ectodermal tissue in the formation of free digits. Our results show that the earliest and most conspicuous structural alterations of the limb induced by JGB take place in the ectodermal tissue and specially at the epithelial–mesenchymal interface.

MATERIALS AND METHODS

We employed White Leghorn chick embryos. A total dosage of 8·5 μg or 10 μg of Janus Green B (JGB) (Gurr) in 0·1 ml of saline solution was injected into

the amniotic sac of embryos of 6 or 6·5 days of incubation (stages 29 and 30 of Hamburger & Hamilton, 1951). Control embryos injected with the same volume of saline were also studied. The legs of 100 control and 162 experimental surviving embryos were studied by the following techniques:–

(a) Neutral red vital staining

The necrotic areas were mapped *in ovo* by vital staining with neutral red following the method of Hinchliffe & Ede (1973) in control and experimental embryos of 7, 7·5, 8 and 8·5 days of incubation (stages 31 to 35 of H.H.).

(b) Whole-mount cartilage staining

For this purpose we employed embryos of 8, 9, 9·5, 10 and 10·5 days of incubation (stages 33 to 37 of H.H.) fixed in Bouin's fluid. The limbs were dissected off, stained with alcian blue 8GX (Gurr) following the technique of Ojeda, Barbosa & Bosque (1970) and cleared in methyl salicilate to reveal the skeletal elements of the foot.

(c) Semithin sections and TEM

The leg buds of embryos ranging from stages 31 to 37 sacrificed at 12 h intervals were fixed in 2·5% glutaraldehyde in 0·1M-cacodylate buffer (pH 7·2) for 4 h, they were then rinsed in buffer solution in which small fragments of the interdigital and digital zones of the foot were dissected free and postfixed in 1% osmium tetroxide. The selected fragments were dehydrated in a graded series of acetone and propylene oxide and embedded in Araldite. To ensure that the specimens would be sectioned longitudinally they were embedded in flat capsules and carefully oriented under the binocular dissecting microscope. Serial semithin sections were cut with a LKB ultratome III and stained with 1% toluidine blue. Ultrathin sections of selected areas were then made, mounted on uncoated copper grids, stained with uranyl acetate and lead citrate and examined with a Zeiss EM 10C electron microscope.

(d) SEM

The feet of embryos sacrificed and fixed as above were dehydrated in a series of acetones and dried by the critical-point method. The specimens were then gold-sputtering coated and viewed in a Philips SEM 501 electron microscope.

At least five experimental and three control embryos of each stage were studied by each of the technical procedures.

RESULTS

JGB administration to both 6 and 6·5 days chick embryos results in malformations of the foot at a frequency ranging from 30% of the surviving embryos at a dosage of 8·5 μg to 50% of the surviving embryos at a dosage of

10 μg. The malformed feet can already be identified at 7·5 and 8 days of incubation. As can be seen in Fig. 1, the interdigital commissures appear less developed than in controls (Fig. 2). The interdigital spaces are narrower and digit III tends to be curved towards the tip of digit IV. All these features give the malformed foot a rounded contour which contrasts with the dentate shape of the foot of normal embryos. Whole-mount cartilage staining shows ectopic nodules of cartilage in the interdigital spaces (Fig. 3) as early as day 8 of incubation. From day 9·5 of incubation onwards the malformation achieves its maximum intensity and consists of i) presence of interdigital membranes (Fig. 4); ii) hypophalangy (Fig. 5) and iii) presence of ectopic nodules of cartilage in the interdigital membranes (Figs. 5, 6). These malformations display a variable intensity and are most often present in digits III and IV and their interdigit.

Cell death

Vital staining with neutral red reveals a significant reduction of the interdigital necrotic areas in the experimental as compared to the control embryos (Fig. 7). The diminution of the necrotic areas is especially prominent from day 7·5–8 of incubation when the leg shows an abnormal shape with significant reduction in size of the interdigital spaces. Semithin sections showed a diminution of both isolated dying cells and macrophages, with no apparent difference in the relative amount of each.

Dying cells and macrophages were also present in the mesenchymal and ectodermal tissue of the older limbs but they do not seem to achieve a high-enough intensity to be clearly revealed by vital staining.

Fig. 1. Low-magnification SEM view of a malformed limb bud at day 8 incubation. The digits appear curved. The interdigital grooves are poorly developed showing ectodermal bulging zones. Note also the lack of interdigital commissures. Bar = 0·25 mm.

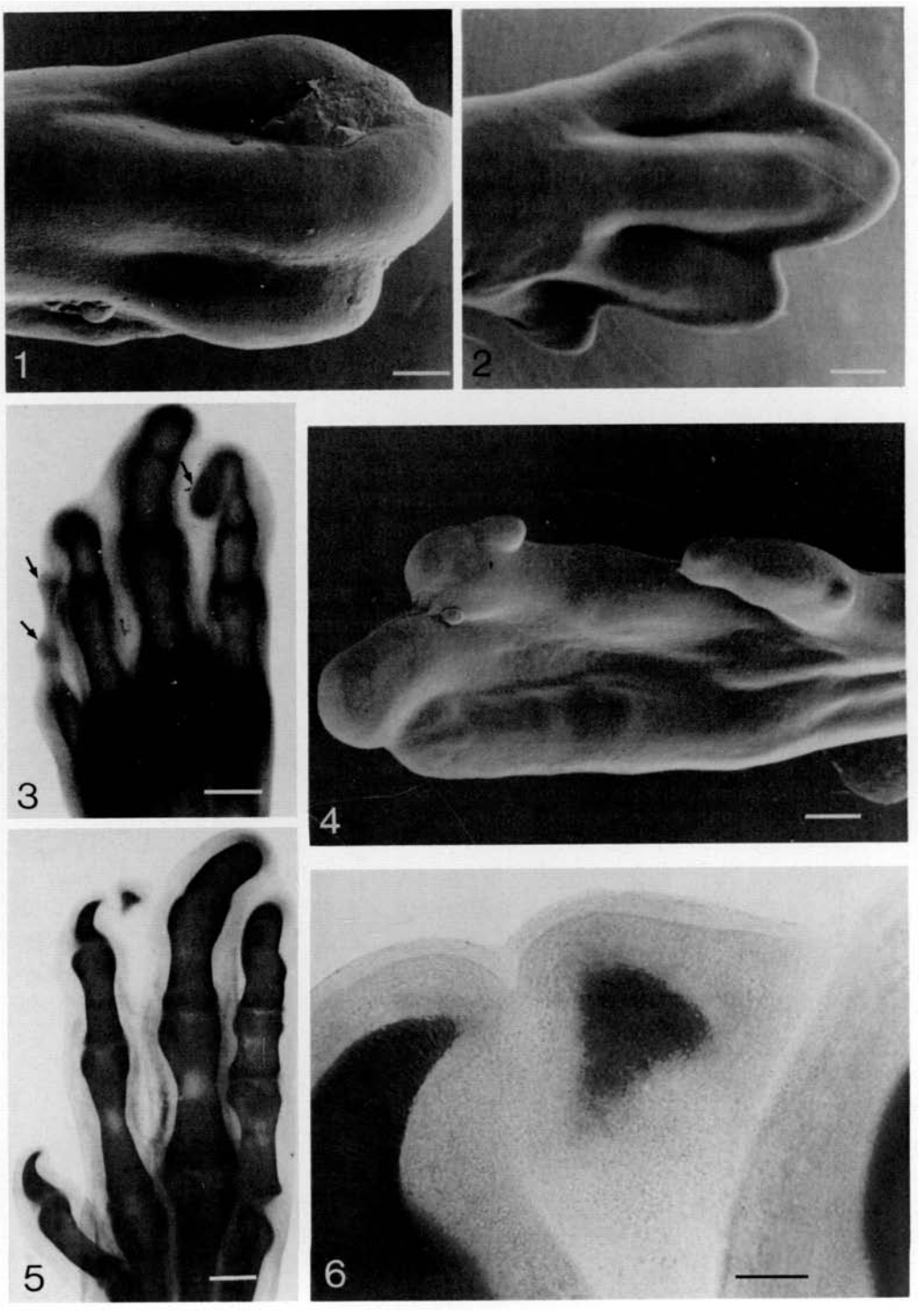
Fig. 2. Control leg bud at day 8 of incubation. The digits are straight. The interdigital grooves are deep and interdigital commissures are prominent. Bar = 0·3 mm.

Fig. 3. Whole-mount cartilage staining of a malformed foot of day 8 of incubation. Note the presence of ectopic cartilages (arrows) in the interdigital spaces I–II and III–IV. Bar = 0·3 mm.

Fig. 4. Low-magnification SEM view of a malformed foot at day 9·5 of incubation. Note the reduced extension of the remaining interdigital tissue. Bar = 0·3 mm.

Fig. 5. Whole-mount cartilage staining of a malformed foot at day 10·5 of incubation showing hypophalangy and interdigital membranes. Digits III and IV have only three phalangy. Note also the presence of a small ectopic cartilage in the interdigit II–III. Bar = 0·3 mm.

Fig. 6. Detailed view of the ectopic cartilage showed in Fig. 5. Bar = 0·1 mm.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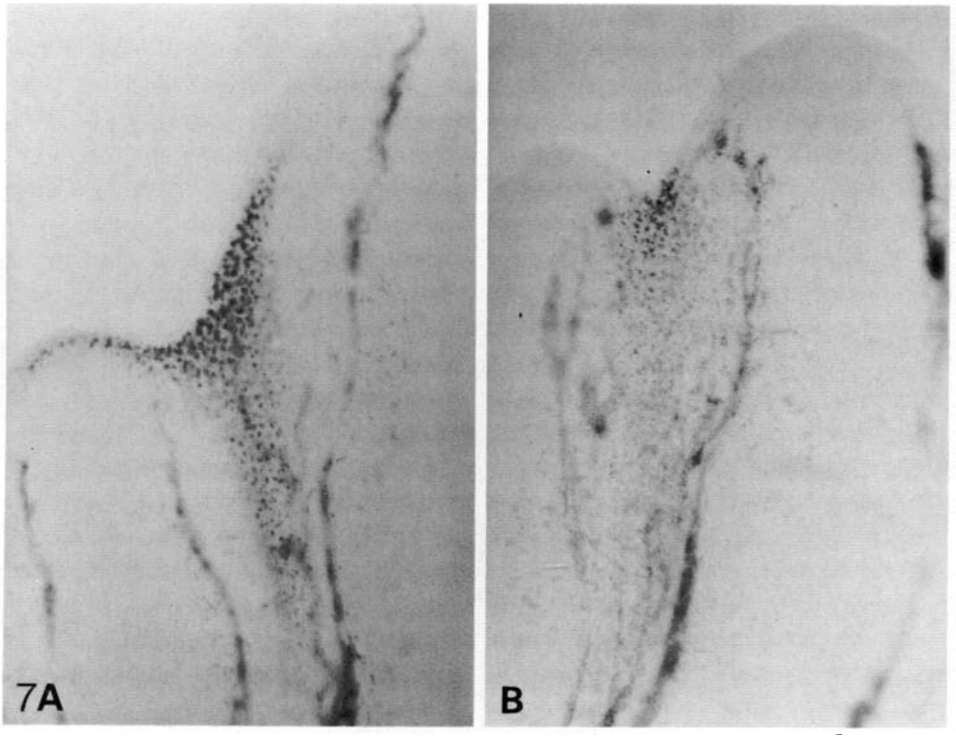

Fig. 7. Vitally stained control (A) and experimental (B) feet showing the pattern of interdigital cell death in the interdigit III-IV at day 8 of incubation. Note that the experimental foot is already malformed.

SEM

Scanning electron microscopy examination reveals significant surface changes in the malformed legs. At earlier stages (prior to day 9·5) the alterations comprise a disappearance of the AER as early as day 7, and the presence of bulges of the ectodermal tissue and zones denuded of ectodermal cells (Figs. 8, 9, 10). As can be seen in Fig. 9, the ectodermal bulging zones appear covered by very irregular ectodermal cells. Most of them are rounded and rich in microvilli, others are elongated and display a smooth surface.

Fig. 8. Low-magnification SEM view of the interdigital space III–IV of an experimental foot at day 8·5 of incubation. No interdigital commissure is present. A prominent bulge of ectodermal cells is present in the interdigital space. Bar = 0·1 mm.

Fig. 9. Detailed view of the bulge of ectodermal cells showed in Fig. 8. Note the irregular shape of the cells. Most of the cells are rounded and rich in microvilli but cells flattened and showing a smooth surface are also present (arrows). Magnification bar = 25 μm.

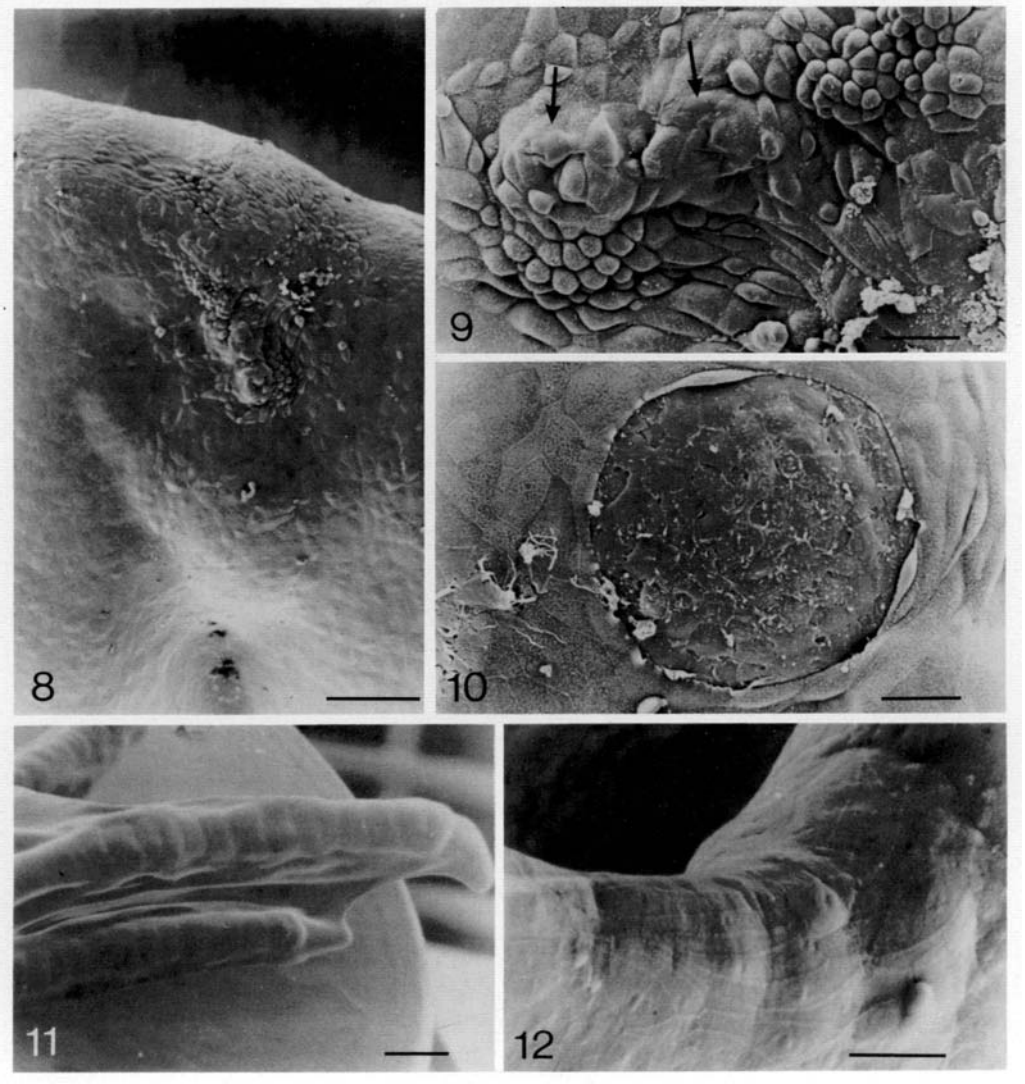

Fig. 10. SEM micrograph of an interdigital membrane of a malformed foot showing a rounded zone devoid of ectodermal cells. Day 8·5 of incubation. Magnification bar = 25 μm.

Fig. 11. Low magnification SEM view of an experimental foot at day 10·5 of incubation showing a prominent interdigital membrane in the interdigit III–IV. Magnification bar = 0·3 mm.

Fig. 12. Detailed view of the commissure of the interdigital space III–IV showed in Fig. 11. Note the elongated shape of the commissural ectodermal cells with the long axis oriented transversely to the interdigit. Magnification bar = 20 μm.

Detaching degenerating cells and cell fragments are also present at the bulging zones. All these alterations are more frequent in the interdigital spaces but they can also be observed in the digital zones.

After day 9·5–10, once the interdigital membranes are clearly observed (Fig. 11) these alterations are very scarce or absent and the ectodermal surface tends to show a normal appearance. It is interesting to note, however, that the commissural ectodermal ridges typical of advanced stages of interdigital tissue regression (Hurle & Colvee, 1982) are not observed in the interdigital commissures with complete syndactyly (Figs 11, 12). In these zones the cells appear elongated with the long axis oriented transversely to the commisure (Fig. 12).

Semithin sections

Alterations in the structure of the experimental legs are detected by semithin sections from day 7 of incubation throughout the whole period covered by this study. The alterations were especially prominent in the epithelial layer and epithelial–mesenchymal interface of the marginal zone of the limb. Although alterations can be observed in the digital zones, they are more abundant in the interdigital spaces. For comparison with normal embryos we shall refer here to the third interdigital space of the experimental embryos. Control embryos did not show in any case comparable alterations and their structure will not be described here due to their complex developmental changes. A detailed description of the structural evolution of the third interdigital space in normal embryos has been previously published by us (Hurle & Fernandez-Teran, 1983).

With the exception of the presence of mesenchymal condensations and nodules of cartilage in the interdigital spaces mentioned above, the most conspicuous alterations in all stages are present in the ectoderm. The ectodermal tissue shows a significant enlargement of the intercellular spaces and zones of disintegration of the basal surface. In these zones the basal ecto-

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Fig 13 to 18. Longitudinal semithin sections of the interdigit III–IV of experimental embryos showing different degrees of ectodermal alteration. Bar = 25 μm.

Fig. 13. AER showing a prominent irregularity of the basal surface (arrows) at day 7 of incubation.

Fig. 14. Extensive flattening of the AER at day 7·5 of incubation. Note the presence of macrophages at the epithelial–mesenchymal interface. The mesenchymal tissue appears condensed showing a small foci of isolated dying cells and macrophages.

Fig. 15. Flattening of the marginal ectoderm at day 8·5 of incubation. A remnant of basal lamina was detected by TEM following the course indicated by the arrows (see also Fig. 24).

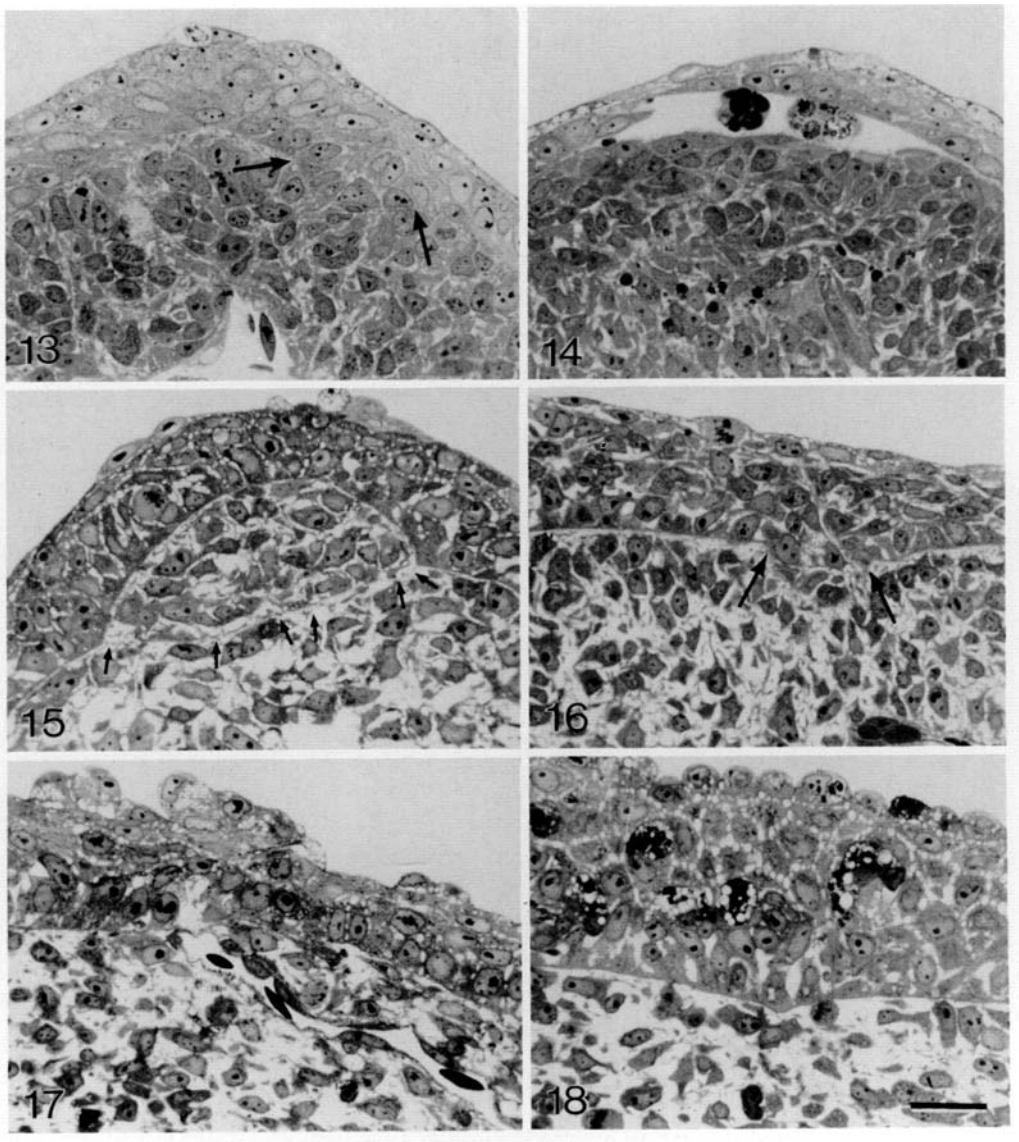


Fig. 16. Focal area of the marginal ectoderm in which the basal ectodermal cells are being detached into the mesenchymal tissue (arrows). Day 8·5 of incubation.

Fig. 17. Zone of contact between a blood vessel and the abnormal ectodermal tissue. Day 9·5 of incubation.

Fig. 18. Ectodermal tissue at day 10 of incubation showing degenerating cells and enlarged intercellular spaces.

dermal cells appear to be in the process of detachment towards the mesenchymal tissue. Ectodermal cells are also observed in the process of detachment into the amniotic fluid. At day 7–7·5 the alterations are especially frequent in the AER. As can be seen in Fig. 13, the ridge appears rather flattened showing zones of rupture of the basal lamina. A more intense alteration is illustrated in Fig. 14 in which the ridge is completely lost, showing dying cells and macrophages in the enlarged epithelial–mesenchymal interface. At days 8, 8·5 and 9 of incubation the marginal ectoderm appears reduced in thickness (Fig. 15). As can be seen in Fig. 16 the ectodermal basal surface shows zones in which the basal ectodermal cells appear projected towards the mesenchymal tissue. In some cases (Fig. 17) blood vessels reached the disintegrated basal surface of the ectodermal tissue. This feature was never observed in control embryos. From day 9·5 onwards, ruptures of the ectodermal basal surface are scare. The ectodermal tissue appears thickened, showing a squamous appearance with frequent dying cells and macrophages (Fig. 18). The large deposits of collagenous extracellular material typical of the marginal mesenchymal tissue of the normal embryos at these stages (Hurle & Fernandez-Teran, 1983) were never observed in embryos with complete syndactyly.

TEM

In addition to the mitochondrial alterations of the epithelial and mesenchymal cells described in previous studies (Pautou, 1978; Pautou & Kieny, 1971) our observations reveal very early structural alterations of the ectodermal tissue and especially of the epithelial–mesenchymal interface.

In the ectodermal epithelium the increase in the intercellular spaces observed in the semithin sections appears to be due to a reduction in the area and number of cell junctions. The large and abundant gap junctions typical of the AER disappear and only occasional small gap junctions are observed (Fig. 19). While in the normal embryos gap junctions disappear when the AER flattens out (Hurle & Fernandez-Teran, 1983), in the malformed embryos these small gap junctions are observed throughout the period studied. The alterations of the epithelial–mesenchymal interface are also confirmed by TEM. The basal lamina appears discontinuous or detached from the basal surface of the ectodermal cells (Figs. 19, 20). The morphology of the basal ectodermal cells in the zones of basal lamina alteration are compatible with a process of detachment of the ectodermal cells and subsequent movement towards the marginal zone of the mesenchymal core of the limb. As can be seen in Fig. 21, in some instances the borderline between the epithelium and mesenchyme is lost. On other occasions, some of the basal ectodermal cells appear located in the mesenchymal tissue (Fig. 22). Basal ectodermal cells are also observed located in zones limited by branches of a duplicated basal lamina (Fig. 23). In some instances the whole marginal ectoderm is devoid of basal lamina and remnants of basal lamina are observed within the marginal mesen-

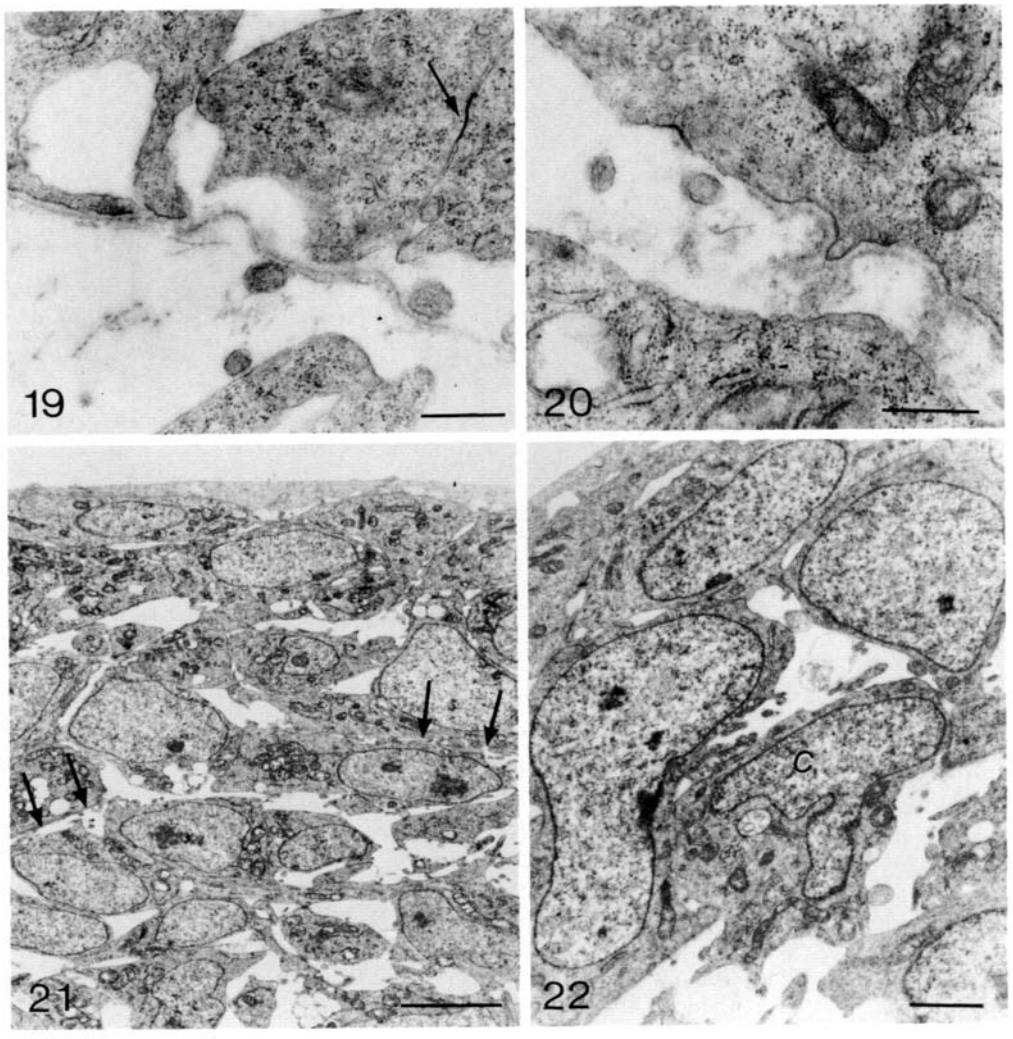


Fig. 19. TEM micrograph of the marginal ectoderm of the interdigit III–IV of an experimental embryo at day 8 of incubation. The basal lamina appears in course of detachment from the basal ectodermal surface. Arrow shows a gap junction between the basal ectodermal cells. Bar = 0·5 μm.

Fig. 20. TEM micrograph showing a zone of interruption of the basal lamina of the ectodermal epithelium in an experimental embryo at day 8 of incubation. Bar = 0·5 μm.

Fig. 21. Low-magnification TEM micrograph of the ectodermal tissue of the interdigit III–IV of an experimental embryo at day 8 of incubation. The limits between the epithelium and the mesenchyme are not distinguishible. Arrows show zones in which the remnants of the basal lamina were observed. Bar = 5 μm.

Fig. 22. TEM micrograph showing a basal ectodermal cell (*c*) in course of detachment into the mesenchymal tissue. Interdigital space III–IV of a 8·5 experimental embryo. Bar = 2 μm.

chymal cells (Fig. 24). As can be seen in Fig. 24, in these cases the detached basal lamina appears apposed to small zones of the mesenchymal cells located distally to it.

DISCUSSION

Our observations have confirmed most of previous studies on the effect of JGB on limb morphogenesis and also provide significant new information which may be valuable for a better understanding of limb development.

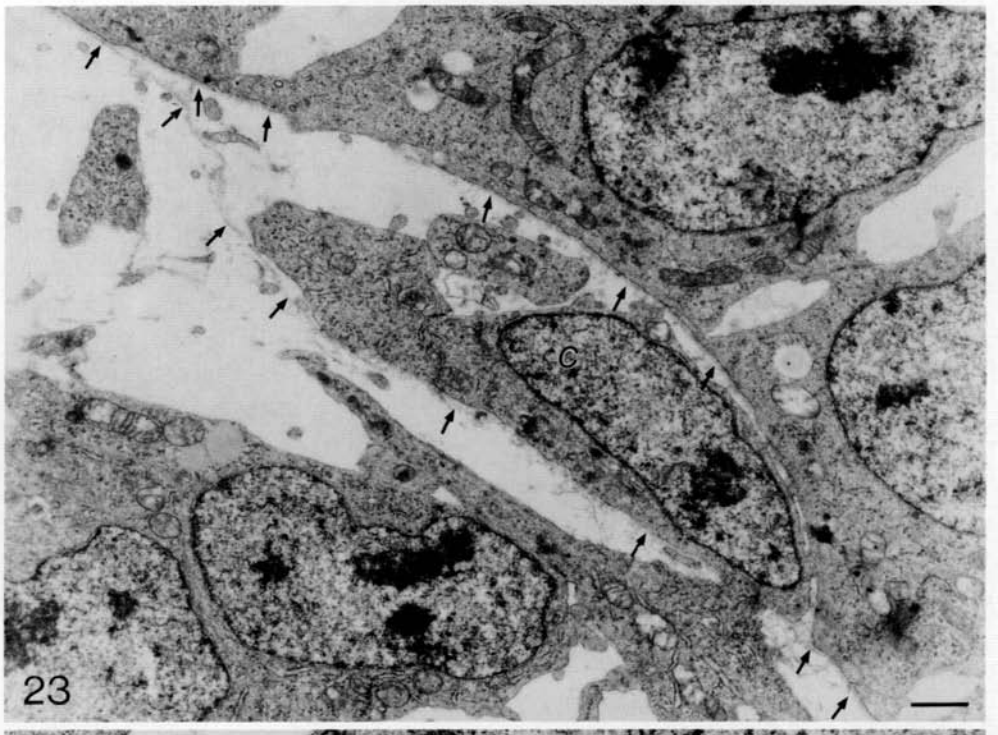
As has been described in previous studies, at the cellular level the most significant alteration produced by JGB takes place in the mitochondria (Baba, 1970; Fallon, 1972b; Pautou & Kieny, 1971). This alteration seems to be due to the attachment of the JGB to a specific lipoprotein of the mitochondria, thus interfering with cellular respiration (Braun, 1954; 1964).

At the tissue level, two main alterations were observed: i) disruption of the epithelial tissue and the epithelial–mesenchymal interface, and ii) reduction or absence of interdigital cell death. Two main questions are raised by these results: which of these tissue alterations can account for the different aspects of the abnormal limb development induced by JGB?; might they be related to each other?

The epithelial alteration is the most precocious phenomenon observed by us after JGB administration. The alteration takes place preferentially in the AER zone, and involves a dramatic alteration of the epithelial–mesenchymal interface. It is well known that the AER regulates limb outgrowth by maintaining the subridge mesoderm in an undifferentiated growing state (Zwilling, 1968). Furthermore, this inductive effect seems to depend on the structural and compositional characteristics of the subridge extracellular matrix (Kosher & Savage, 1981; Sawyer, 1982). These facts may explain the hypophalangy typical of the malformed limb, as suggested previously (Pautou & Kieny, 1971). A significant feature observed by us and unreported in previous studies, is that the flattening of the AER induced by JGB is accompanied by a reduction in the number and extension of gap junctions. These junctions are distinctive structures of the AER (Fallon & Kelley, 1977) which disappear in chick mutants with defective limb outgrowth (Sawyer, 1982) as well as during normal regression of the AER (Hurle & Fernandez-Teran, 1983). But as

Fig. 23. TEM micrograph showing the presence of a cell (*c*) located under the ectodermal tissue in a zone in which the basal lamina appears duplicated. Arrows show the arrangement of the two branches of the basal lamina. Experimental embryo at day 8 of incubation. Bar = 1 μm.

Fig. 24. TEM micrograph of one of the segments indicated by arrows in Fig. 15, showing remnants of the basal lamina located between the mesenchymal cells. Arrow shows a zone of apposition of the basal lamina-like material to a mesenchymal cells. Experimental embryo at day 8·5 of incubation. Bar = 0·5 μm.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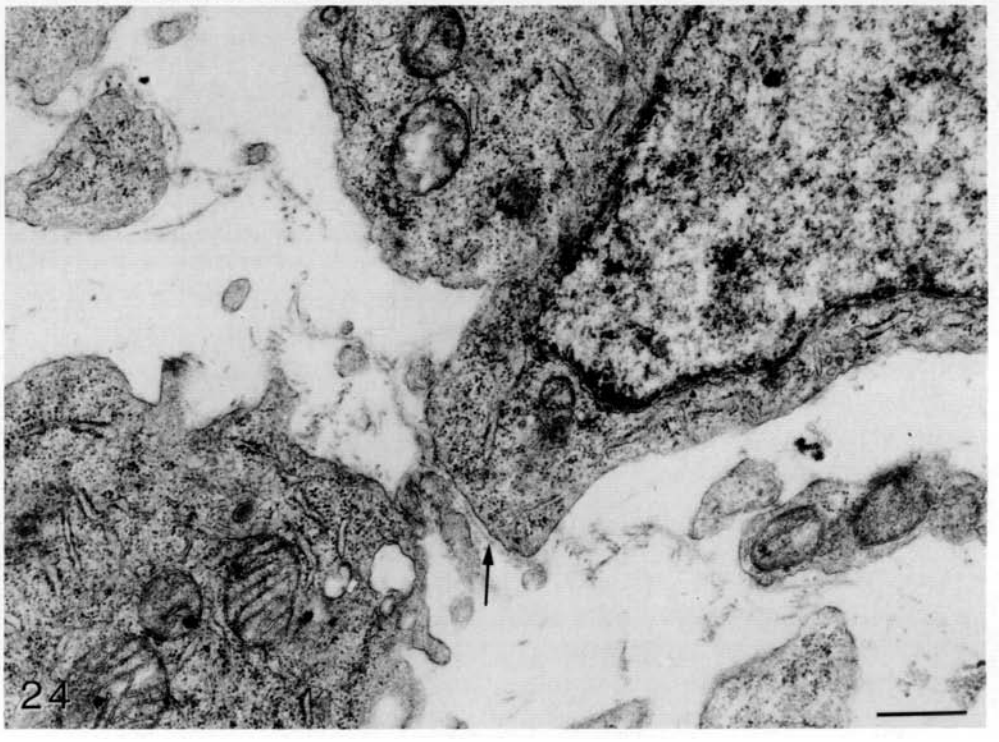
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distinct from the normal regression of the AER, the regression of the AER induced by JGB does not produce a complete disappearance of the gap junctions. This is a particularly interesting feature. It has been suggested that in the chick limb the onset of interdigital cell death might be related to a sharp interruption of the inductive effect of the AER on the subridge mesoderm (Kelley & Fallon, 1983; Hurle & Fernandez-Teran, 1983) which is accompanied by a rapid disappearance of gap junctions within the ridge cells. This hypothesis is supported by the fact that the duck leg, which has a minor intensity of interdigital necrosis, seems to show a slower disappearance of gap junctions in the regressing AER (Hurle & Fernandez-Teran, 1984). All these facts suggest that the reduction or absence of interdigital cell death might well be due to an alteration of the epithelial–mesenchymal interaction rather than to a direct effect of the JGB on the mesenchymal cells scheduled to die or on the phagocytic reaction within the necrotic area as proposed previously (Deleanu, 1965; Menkes & Deleanu, 1964; Pautou & Kieny, 1971). Our hypothesis is also supported by the fact that JGB is a mitochondrial toxin which in other systems not only does not inhibit cell death but actually causes it (Menkes *et al.*, 1966; Menkes, Sandor & Ilies, 1970; Minclea & Arcan, 1966; Pavkov & Mirkov, 1968). Furthermore, in the malformed limbs studied by us the ratio of macrophages to dying cells seems to be similar to that in normal embryos.

An interesting feature observed by us is that the disruption of the ectodermal tissue is accompanied by detachment of the ectodermal cells towards the underlying mesenchymal tissue. There are examples of developing organs, such as heart, neural crest or the somites, in which a transformation of the epithelial cells into free mesenchymal cells takes place (Markwald, Fitzharris & Manasek., 1977; Nichols, 1981; Solursh, Fisher, Meier & Singley, 1979). There is evidence showing that this phenomenon depends on the nature of the underlying extracellular matrix (Nichols, 1982). Furthermore it has been hypothesized that the maintenance of the epithelial tissues is a dynamic phenomenon which depends on the interaction between the epithelial cell membranes and the extracellular matrix (Hay, 1982). Our observations of a transformation of the ectodermal cells of the limb bud into free mesenchymal cells in zones in which the junctions between ectodermal cells are reduced and the basal lamina is disrupted, supports this hypothesis. The role of the mesenchymal cells derived from the marginal epithelium of the limb bud cannot be ascertained from our observations. However, it does not seem too speculative to suggest that they might be involved in the maintenance of the interdigital tissue and the eventual formation of interdigital membranes.

Another significant observation derived from our study is that when interdigital cell death is inhibited, the mesenchymal cells can follow a developmental programme similar to that of their neighbouring cells programmed to form digits. Similar results were observed by Saunders & Fallon (1967) in

studies of the posterior necrotic zone of the developing limb. These necrotic cells are located in the prospective elbow area and when cell death was inhibited by grafting the prospective necrotic cells into host embryos prior to their definitive determination to die, they survived and formed cartilage nodules covered by skin and feather germs in a pattern similar to that found on the elbow. In our study the interdigital tissue in many instances formed cartilage nodules covered by skin with scales as occurs in the digits. This fact supports the view that the cells scheduled to die can be diverted to continue life and differentiate according to their position. This tends to confirm the hypothesis that cell death in the developing limb plays the role of limiting the number of mesenchymal cells available to form skeletal elements (Hinchliffe, 1981). An alternative explanation is that the regression of the AER produced by JGB could induce the differentiation of the interdigital mesenchymal cells into cartilage, thus arresting the program for death. In this respect it must be mentioned that the AER has an inhibitory effect on chondrogenesis (Kosher, Savage & Chang, 1979; Solursh, Singley & Reiter, 1981) which presumably disappears under our experimental conditions.

Finally, in limbs with total syndactyly, we did not find the structural changes typical of later stages of interdigital tissue regression (Hurle & Fernandez-Teran, 1983; 1984). This supports the hypothesis that the disappearance of the interdigital membranes requires the participation of all of its tissue components.

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