Early changes in limb buds of chick embryos after thalidomide treatment

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Since the first observations of hypoplastic and aplastic thalidomide deformities in infants (McBride, 1961; Lenz, 1962), the literature on this subject has grown to many hundreds of communications. Experimental investigations in almost all cases have been undertaken to show whether thalidomide and its metabolites have any teratogenic effects in experimental animals. Numerous review papers are available on this subject, e.g. Giroud, Tuchmann-Duplessis & Mercier-Parot (1962), Somers (1963), and Salzgeber & Wolff (1964).

Chick embryos did not seem for some time to be suitable for experimental production of typical thalidomide deformities. However, Kemper (1962a, b), Yang, Yang & Liang (1962), Boylen, Horne & Johnson (1963) and Leone (1963) have shown that thalidomide can produce a whole range of ectromelian deformities provided that it is introduced into the egg at a particular period of embryonic development. Certain controversy was created when Williamson, Blattner & Lutz (1963) demonstrated that certain insoluble and otherwise inert substances such as powdered glass, colloidal alumina and colloidal clay, when introduced into the amniotic cavity in chick embryos, caused thalidomide-like malformations.

More recently, Salzgeber & Salaun (1963a, b, 1965) established that thalidomide produces in the chick essentially the same kind of malformations as it does in human and rabbit embryos.

The present report gives an account of an investigation of short-term changes in chick embryos treated with thalidomide. This approach, if successful, would help to elucidate the mode of action of thalidomide on limb primordia when they are in the sensitive period of development.

MATERIAL AND METHODS

Thalidomide suspensions

Thalidomide (Distillers Co. Ltd.), well ground and dry-sterilized at 100 °C, was suspended in thin egg-white (10 or 20 mg/ml of medium). The egg-white was taken from non-experimental infertile fowl eggs. To these suspensions penicillin and streptomycin (2.5 mg of each per ml) were added.

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Treatment of chick embryos

Fowl eggs (Brown Leghorn or Chunky Chick) were incubated at 38.5 °C for 60–64 h until the stage 17 or 18 of Hamburger & Hamilton (1951) was reached. The eggs were then opened through the air space by removing about 1–2 cm² of the calcareous shell together with the outer shell membrane. After the inner shell membrane had been pierced with a short-bevel hypodermic needle, 0.2 ml of thalidomide suspension was introduced into the egg-white in close proximity to the embryo, but not into the amniotic cavity. The same amount of suspension was also injected into the yolk sac just under the blastoderm in some eggs. These eggs were injected with a total of 0.4 ml of suspension. Since suspensions were used in two concentrations and some eggs were injected only in the egg-white and some in both egg-white and yolk, there were essentially four kinds of experimental embryos. The results obtained in all four experimental groups will be reported together, since embryos with all degrees of injury were found in each group. The degree of injury does not seem to be directly dependent upon the dose of thalidomide or upon the method of its application.

Control eggs were injected with thin albumen containing the same amount of calcium carbonate instead of thalidomide and with the same amounts of penicillin and streptomycin. Calcium carbonate has a low solubility in water like thalidomide and is probably harmless to avian eggs as it constitutes up to 98.5 % of the egg shell.

After injections the eggs were sealed with Parafilm (Lindsay and Williams, London) and incubated for 24 h at 38.5 °C. For further investigations only those embryos were selected which showed heart beat and were between stages 21 and 25 at time of fixation (Hamburger & Hamilton, 1951).

Light- and electron-microscopy techniques

For light-microscope observations the embryos were prepared according to the technique described previously (Jurand, 1965).

For electron-microscope investigations both osmic acid fixation (Jurand, 1962) and glutaraldehyde fixation followed by 1 % osmic acid postfixation were used. In the latter case 5 % glutaraldehyde solution was buffered with cacodylate buffer at pH 7.2 (Plumel, 1948) and 0.1 mg of calcium chloride was added for each ml of fixative. The embryos were fixed for 4 h in the glutaraldehyde solution at 2–4 °C. After rinsing with chilled buffered 7.5 % sucrose solution (3 times, 15 min each time) the forelimb buds were dissected and postfixed with 2 % osmic acid fixative (Jurand, 1962). Further preparation was done as described previously (Jurand, 1965).

To localize the acid phosphatase activity in the endothelial cells of the limb-bud axial artery, the procedure of Gomori (1952), as adapted to electron microscopy by Holt & Hicks (1962), was applied (for detailed description see Jurand, 1965).
**RESULTS**

In normal conditions at stages 21–25, i.e. after 64–68 h of incubation, the axial artery is usually narrow and unbranched. Early injuries after thalidomide treatment appear in about 40% of the embryos as distinct dilation of the limb-bud axial artery with or without necrotic changes in the surrounding mesoblast tissue. These changes, arbitrarily classified here into three groups, are easily observable with a low-power dissecting microscope. They are particularly visible in dehydrated specimens cleared with methyl benzoate, in whole mounts cleared in xylol and embedded in Canada balsam and also in specimens cleared by embedding in Araldite for electron-microscope purposes.

In histological sections taken from limb buds with distinct dilations of the axial artery, the endothelium of the blood vessels is very thin. The vessels are often convoluted, forming diverticula filled with blood corpuscles (Plate 1, figs. A, B). This kind of damage is classified as first-degree injury. In more affected limb buds (Plate 1, fig. C), the dilations of the axial artery are wider and a considerable number of necrotic cells are found in the mesoblast tissue adjacent to the dilated part of the blood vessel (second-degree injury).

In extreme cases the vast majority of the mesoblast tissue is completely destroyed, leaving extensive empty spaces without any blood vessels (third-degree injury). In these cases, however, there is usually a small concentration of more or less healthy looking mesoblast cells in the dorsal part of the limb bud just below the dorsal epiblast and extending into the area below the apical ectodermal ridge (Plate 1, fig. D).

Usually those embryos in which the limb buds show more prominent injury (second or third degree) are also retarded in general development.

Regardless of the degree of injury, the epiblast (together with the apical ectodermal ridge) never showed any visible abnormality, for instance in the form of discontinuities or necrosis.

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**EXPLANATION OF PLATES**

Abbreviations on Plates: cly., cytolsome; e.c., endothelial cell; lu., lumen of the axial artery; ly., lysosome; m.c., mesoblast cell; n., nucleus.

**PLATE 1**

Fig. A. Sagittal section through a normal chick wing bud at stage 23. × 80.

Fig. B. Prominent dilation of the axial artery (first-degree injury), stage 22. × 80.

Fig. C. Scattered necrosis around the dilated artery (first-degree injury), stage 21. × 80.

Fig. D. Section through an embryo at stage 20, showing extensive damage of the limb bud mesoblast. Note the concentration of normal mesoblast cells in the dorsal and marginal area of the limb bud. × 80.

Fig. E. Mitochondria in a control endothelial cell. × 23000.

Fig. F. Swollen mitochondria in the endothelium after treatment (first-degree injury). × 23000.
Electron microscopy

Electron-microscope investigations were concerned mainly with the ultrastructure of the endothelial cells of the axial artery and of the adjacent mesoblast cells. The epiblast with the apical ectodermal ridge were also examined, but in these parts no divergence from normal ultrastructural patterns was observed.

Depending on the degree of injury, the endothelial cells show more or less distinct changes at the ultrastructural level. In the limb buds with markedly dilated axial artery but without extensive necrotic changes in the mesoblast (i.e. first-degree injury), the mitochondria in endothelial cells show considerable changes. In comparison with the controls, the affected mitochondria are markedly increased in size, rounded up, often partly or completely vacuolated and contain few cristae. The control mitochondria are elongated, with a much more electron-dense matrix and have the usual appearance of the metazoan type (Plate 1, figs. E, F; Plate 2, figs. A–C).

In many cases of first-degree injury the alteration of mitochondrial structure was confined to those in the endothelial cells; the adjacent mesoblast cells contained mitochondria of the same appearance as those in control endothelial cells (Plate 2, fig. B).

Also observed in the limb buds with first-degree injury was an increase in number of lysosomes in the endothelial cells. They formed groups of up to ten in one section and were frequently found near the Golgi apparatus (Plate 2, figs. C, D).

Morphologically, at least three types of lysosomes can be distinguished: (1) homogeneous type covered by a single membrane, (2) multivesicular body type (see Plate 4, fig. B), and (3) cytolysome type. The last type is usually found in more affected limb buds (Plate 2, fig. E).

In control endothelial cells only single droplets of the homogeneous type of lysosome were observed, and these very seldom.

In affected endothelial cells the lysosomes are often found close to vacuolated mitochondria, and in these cases the vacuole inside the mitochondrion is usually adjacent to the side where the lysosome is lying (Plate 3, fig. A). In some cases lysosomes, often containing myelin-like figures, were found embedded inside the injured mitochondria (Plate 3, fig. B).

Plate 2

Fig. A. Vacuolated mitochondria. × 22000.

Fig. B. Swollen mitochondria confined to the endothelial cell (e.c). Below, a mesoblast cell (m.c.) with normal mitochondria. × 15000.

Fig. C. Endothelial cell with Golgi apparatus and adjacent primary and secondary lysosomes. × 33000.

Fig. D. Group of lysosomes of various types in an endothelial cell. × 29000.

Fig. E. Cytolysomes in an endothelial cell. × 37000.
Another feature of the affected endothelium in these limb buds is the vacuolation of the cytoplasm. The vacuoles are often seen to be formed by dilation of endoplasmic reticulum cysternae (Plate 3, fig. D).

In limb buds which show the two higher degrees of injury the endothelial cells also display other characteristic properties. At first, when glutaraldehyde fixative is used, the cytoplasm of the endothelial cells becomes much more electron-dense than that of the adjacent mesoblast cells (Plate 3, fig. A). This difference is due to the increased number of ribosomes per unit volume; they are closely packed and uniformly fill the cytoplasm. In adjacent mesoblast cells the ribosomes are arranged in typical polysome groups scattered fairly loosely throughout the cytoplasm. Each polysome group consists of 6–9 units. In control limb buds there is no such difference in electron density between endothelial and mesoblast cells, although the same polysome arrangement of the ribosomes is also obvious only in the mesoblast cells (Plate 3, fig. C).

A subsequent ultrastructural feature of the affected endothelial cells is the formation of vesicular projections by these cells. They extend deep into the lumen of the blood vessel and are covered on the outside by the cell membrane and on the inside by a kind of vacuolar membrane (Plate 4, fig. A). In many cases such projections appear to lie loosely in the lumen of the blood vessel, but this is due to the fact that the point of attachment does not lie at the level of the section.

It was observed that these loop-like profiles of the vesicular projections, if sectioned at the point of attachment, have at the base a lysosome embedded in the endothelial cell (Plate 4, fig. B). This suggests that the lysosomes take part in the formation of these projections.

Frequently the vesicular projections are found to be formed by extremely thin endothelial cells (down to 50 m), and very often the continuity of the endothelium is broken at the point where such a vesicular projection is attached. Thus it may be that this is why the endothelium is weakened in the affected limb buds, and that in those with the higher degrees of injury this is the way discontinuities are formed in the endothelial lining (Plate 4, fig. C).

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**Plate 3**

Fig. A. In the upper part an endothelial cell (e.c.) with two mitochondria. Close to one of them a lysosome. Below, a mesoblast cell (m.c.). Note the difference between the electron density of the two cytoplasm and between the arrangement of their ribosomes. × 24000.

Fig. B. Two mitochondria with myelin figure bodies inside in endothelium. × 25000.

Fig. C. Control endothelium and mesoblast cells (see abbreviations). Note different arrangement of ribosomes and no difference in the electron density of the cytoplasms. Compare with fig. A on this Plate. × 19000.

Fig. D. Vacuolation of the endothelium cytoplasm. Note formation of vacuoles by dilation of the endoplasmic reticulum profiles (arrow). × 33000.
In the limb buds with considerable necrotic changes in the mesoblast adjacent to the abnormal axial artery (second-degree injury), the necrotic cells show enlarged lysosomes, numerous cytoplasmic vacuoles and abnormal mitochondria but the nuclei of these cells do not show any visible necrotic changes (Plate 4, fig. D). Probably the process of necrosis in the cytoplasm precedes any necrotic changes in the nucleus.

Experiments designed to show the localization of the acid phosphatase activity in the affected endothelial cells demonstrated that the Golgi apparatus and the lysosomes contain this enzyme (Plate 5, fig. A). In more affected limb buds, large accumulations of acid phosphatase-positive bodies were found in the cytoplasm of these cells. They can be classified as cytolyosomes or extranuclear necrotic centres (Plate 5, fig. B).

In these experiments it could also be demonstrated that the lysosomes found at the point of attachment of the vesicular projections show a considerable concentration of acid phosphatase (Plate 5, fig. C). This suggests that the formation of these projections is due to the enzymic activity of the lysosomes. The vesicular projections themselves also contain some acid phosphatase, but the specificity of the reaction on membranous structures is rather uncertain.

Similarly, at the points of extreme flattening, the endothelial cells show considerable acid phosphatase activity (Plate 5, fig. D).

**DISCUSSION**

Only after the thalidomide disaster did the methods of experimental embryology and applied pharmacology find more intensive application in the appropriate evaluation of drugs with respect to their potentially dangerous side-effects for developing embryos. The co-operation of embryologists with other specialists in the field is the most advisable way to achieve the necessary progress in overcoming drug-induced embryopathies in man (see Tondury, 1962).

Many of the present discussions concern the methods of examination and the extent to which they should be adopted. Admittedly it is not a simple and easy problem, because of the diversity of the potential side-effects, the differences in sensitivity of various laboratory animals, and because of the difficulties in applying the obtained results to the biological conditions in man. For these reasons there obviously cannot be any standardized testing procedure. It seems,
however, that the first step should be to examine the early effects at the onset of organogenesis rather than to wait for later gross abnormalities. The early effects, if spotted shortly after the administration of the drug to pregnant females or to fertilized fowl eggs, might give quick and reliable indications. The late congenital malformations in newborns do not always reveal all embryopathic properties of the drug under examination, because many embryos, particularly those suffering the most serious injuries, die shortly after treatment.

The results presented here suggest that the primary cause of thalidomide abnormalities is an injury of the endothelial lining of the axial limb artery. This effect by itself may not lead to any developmental abnormalities, but it probably has an influence on the developmental potentialities of the non-vascular part of the mesoblast tissue. This possibility is indicated by the fact that in the embryos with more prominent injury of the axial artery numerous necrotic cells are found in the areas of the future skeleton blastemas.

In extreme cases, the injury leads to a complete cytolysis of the central core and the ventral part of the mesoblast, but always with the exception of the dorsal region near the apical ectodermal ridge (see Plate 1, fig. D). For some reason the mesoblast cells in this region show greater resistance than the rest of the mesoblast. These observations may explain the mechanism of some of the malformations obtained by Salzgeber & Salaün (1965). Taking phocomelia as an example, one can assume that this malformation occurs due to the fact that the cellular material for the formation of the more distal parts of a limb has greater chance to remain undamaged than that of the proximal parts.

The epiblast is undoubtedly insensitive to thalidomide, and all malformations induced by this drug are due to the selective sensitivity of the mesoblast. It is worth mentioning that McBride, whose note published in 1961 was one of the earliest on thalidomide malformations in human babies, correctly interpreted the primary cause of these deformities by emphasizing that it is the mesenchyme, particularly the prospective bone tissue, which is injured.

At the ultrastructural level the thalidomide injury has several aspects. The mitochondria in the endothelial cells appear to be the most sensitive cell organelles and become morphologically abnormal even in the limb buds which show the first degree of injury. This is not surprising, since it has long been known that mitochondria swell and undergo fragmentation in adverse physiological

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**Plate 5**

Fig. A. Positive acid phosphatase reaction in the Golgi cisternae. Note droplets in the course of detachment (primary lysosomes). \( \times 32000 \).

Fig. B. Acid phosphatase-positive large cytolysome. \( \times 31000 \).

Fig. C. Vesicular projection with acid phosphatase-positive lysosome at its base (arrow). \( \times 38000 \).

Fig. D. Extensive acid phosphatase activity between two extremely thinned endothelial cells. \( \times 38000 \).
conditions (Novikoff, 1961; Hartroft, 1964). Regardless of the damaging factor, whether it is dietary deficiency (Hartroft, 1964), starvation (Gansler & Rouiller, 1956), addition of toxic substances to tissue cultures (Frederic, 1958), irradiation with X-rays (Okada & Peachy, 1957; Manteufel & Meissel, 1965), or treatment with drugs, the same syndrome of changes is always observed in mitochondria. They become larger and more spherical, and they show only abnormally short peripheral parts of disrupted cristae. Considerable loss of electron opacity is followed by extensive vacuolation.

The question arises whether the changes in mitochondria imply that thalidomide interferes selectively with the metabolic activities in the endothelium, causing lesions of the vascular system of a developing limb. Indeed, this seems to be the case, because the changes in mitochondria initially are restricted to the endothelial cell. Accordingly, the necrotic changes in the mesoblast outside the axial artery should be regarded as secondary.

Pliess (1962) attempted to explain the mode of action of thalidomide by suggesting that the drug interferes with the normal biochemical processes governing the course of development of mesodermal structures. Kemper (1962) saw in chick embryos some similarities between thalidomide-induced abnormalities and those caused by B-type antivitamins (e.g. 4-deoxypyridoxol, anti-vitamin B<sub>6</sub>). Woollam & Millen (1963) suggested that teratogenic agents in general have an effect on the enzymic processes in cells during organo-genesis and cause anoxia and general disturbance in the intracellular metabolism. In fact, it has been shown that certain thalidomide metabolites (monohydroxy derivatives) affect <i>in vitro</i> the activity of glutamine synthetase and of glutamate dehydrogenase from rat brain (Williams, 1963).

The suggestion that thalidomide interferes with the intracellular metabolism was also made by Villa & Eridani (1963), who observed certain anti-mitotic properties of thalidomide. These properties are not specific for any particular stage of cell division, however, and should be ascribed to a general antimetabolic activity of the drug. They are probably therefore responsible for the general retardation of development.

In the light of the findings reported here, the mitochondrial lesions apparently being the primary effect, anoxia inside the endothelial cells may be the first result, followed by a chain of changes leading to an extensive necrosis of the mesoblast tissue.

On the other hand, it may be that the primary effect is the increase of the lysosome population and that all other changes, particularly those in mitochondria, follow later. This possibility should be taken into account since mitochondria are known to be extremely sensitive to the hydrolytic activity of lysosomal enzymes, particularly to that of phosphatases. When treated <i>in vitro</i> with lysosome preparations they lose their oxidative phosphorylation capacity and show extensive membrane damage. Also, their proteins undergo hydrolysis (see Tappel, Sawant & Shibko, 1963).
The increased number of lysosomes in endothelial cells, and in advanced cases in the mesoblast cells, suggest a potential increase of destructive processes. Necrosis, which is the end result of these processes, is characterized by autodigestion of different parts of the cell, probably by hydrolytic enzymes released from lysosomes (Bessis, 1964).

Lysosomes appear first near the Golgi complexes in the form of small vesicles (primary lysosomes) which are probably produced by pinching off from the Golgi flat cysternae. Due to the toxic activity of thalidomide, they are produced in increased numbers and consequently grow to reach the size of cytolysomes measuring 0.5–2 μ (extranuclear necrotic centres, Jurand, 1965). Necrotic changes in the nucleus (pycnosis, karyorrhexis) follow later as the last stage of cell death. This fact is in agreement with the observation that nuclei separated from cells are much more resistant to the activity of lysosomal enzymes than other cellular organelles (Tappel et al. 1963).

A course of events very similar to that described in this paper was recently reported for duodenal crypt cells in mice after X-irradiation (Hugon & Borgers, 1965). Also, cellular degeneration processes occurring in natural conditions during embryogenesis proceed in a similar way, e.g. in the limb-bud apical ectodermal ridge in the chick and in the mouse (Jurand, 1965).

The higher electron density of the cytoplasm of the endothelial cells in experimental limb buds in comparison with the control endothelium (see Plate 3, figs. A and C) suggests that these cells react specifically to the treatment. The mesoblast cells do not differ from the control cells to any significant degree at the ultrastructural level.

Fig. C on Plate 3 also demonstrates that in normal conditions there is a marked difference between the monosomal arrangement of ribosomes in the endothelial cells and the typically polysomal arrangement in the mesoblast cells. The same situation is also evident in the experimental limb buds (see Plate 3, fig. A). This phenomenon might be of general interest. Specifically, due to their low synthetic activity, the endothelial cells, which are already differentiated, contain ribosomes in the form of monosomes. On the other hand, the mesoblast cells, which are about to begin the process of differentiation into various mesodermal derivatives, contain polysomes consisting of 6–9 units. Apparently these cells have initiated the synthesis of new specific proteins.

**SUMMARY**

Thalidomide in suspension in thin egg-white introduced into chicken eggs preincubated for 64–68 h causes dilation of the axial limb-bud artery in 24 h after treatment. In some cases there are necrotic cells in the mesoblast outside the dilated artery. In the most extreme degree of injury the mesoblast is almost completely destroyed except for a small group of mesoblast cells located in the dorsal area of the limb bud.
At the ultrastructural level the mitochondria in the endothelial cells of the dilated artery become swollen and vacuolated. Lysosomes are found in markedly increased numbers, and the cytoplasm undergoes vacuolation.

The injured endothelial cells become extremely thinned (down to 0.05 \( \mu \)) and form vesicular projections protruding into the lumen of the artery. At these points gaps are found in the continuity of the endothelial cells.

In more heavily affected limb buds both endothelial and mesothelial cells undergo necrosis by accumulation of lysosomes and formation of cytolysomes, while their nuclei still appear to be normal.

Golgi apparatus, lysosomes, and cytolysomes show considerable acid phosphatase activity.

Possible interpretations of the above observations are discussed.

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RESUME

Modifications precoces observées dans les bourgeois de membres après traitement par la thalidomide

La thalidomide, en suspension dans l’albumine, injectée dans des œufs de Poule de 64–68 heures d’incubation, provoque, 24 heures après traitement, la dilatation de l’artère axiale du bourgeon de membre. Dans certains cas, on observe des cellules nécrotiques dans le mésoblaste en dehors de l’artère dilatée. Dans les cas d’altérations les plus intenses, le mésoblaste est presque totalement détruit à l’exception d’un petit groupe de cellules mésodermiques localisées dans la partie dorsale du bourgeon de membre.

A l’échelle ultrastructurale, les mitochondries présentes dans les cellules endothéliales de l’artère dilatée, prennent un aspect gonflé et vacuolisé. On trouve des lysosomes en nombre accru et le cytoplasme devient vacuolaire.

Les cellules endothéliales altérées s’amincissent (inférieur à 0.05 \( \mu \)) et forment des vésicules faisant saillie dans la lumière de l’artère. À ces endroits, les cellules endothéliales sont séparées par des lacunes.

Dans les cas où les bourgeois de membres sont très atteints, les cellules endothéliales et mésothéliales subissent la nécrose par suite de l’accumulation de lysosomes et de formation de cytolysomes, alors que leur noyau paraît encore normal.

L’appareil de Golgi, les lysosomes et les cytolysomes montrent une activité phosphatase acide intense.

La discussion porte sur l’interprétation possible de ces observations.

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