The effect of excess vitamin A on the development of rat embryos in culture

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SUMMARY

Primitive streak stage rat embryos were cultured in serum containing added vitamin A alcohol at concentrations ranging from 0.5 to 20 µg/ml. At the higher concentrations there was an overall growth retardation, and differentiation was inhibited. This was thought to be due to inhibition of DNA synthesis. At 0.5 µg/ml the embryos developed abnormally in a manner similar to that previously seen in vivo. This result, which was supported by an ultrastructural study, provides evidence that the teratogenic effects of vitamin A are due to a direct action on the embryos.

Comparison of embryonic development in vitro at different concentrations of vitamin A with the results obtained in vivo suggests that in the latter, only a very small proportion of the amount injected was reaching the embryos in an active form. The maternal factors involved are discussed, and excess vitamin A is defined as an excess of free and non-specifically bound retinol. The levels of vitamin A which affected differentiating embryos in vitro are lower than the minimum levels previously shown to affect differentiated tissues. This suggests that relatively minor increases in serum levels during pregnancy may cause malformations without affecting the maternal organism. Since raised serum vitamin A levels have been shown to be associated with impaired liver function in man, these factors may be significant in human teratogenesis.

INTRODUCTION

The administration of large amounts of vitamin A to mammals during early pregnancy causes malformations in the offspring (Cohlan, 1953; Giroud & Martinet, 1959). Although this is firmly established, it is not clear whether the teratogenic effect is brought about by a direct action on the embryos, or via an effect on the maternal organism.

In support of the latter, Woollam & Millen (1960) found that the effects of vitamin A were enhanced by cortisone and reduced by insulin and thyroxine, and postulated a mechanism involving interference with maternal carbohydrate metabolism. However, Cohlan & Stone (1961) failed to confirm these results; furthermore they found that parathyroidectomy, thymectomy, or bilateral adrenalectomy themselves had no influence on gross foetal development, nor did they affect the incidence of vitamin A-induced malformations.

In a study of the ultrastructural changes occurring in embryos following the

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maternal vitamin A injection, Morriss (1973a) interpreted the results as supportive evidence for a direct action on the embryo, since the cytological effects were similar to those seen in in vitro studies of mammalian cells and tissues in the presence of excess vitamin A (Glauert, Daniel, Lucy & Dingle, 1963; Daniel, Dingle, Glauert & Lucy, 1966).

This study of the effects of vitamin A on the development of embryos in vitro was undertaken in order to test the hypothesis that vitamin A exerts its teratogenic effects by direct action on the embryos.

A further question concerns the large amounts of vitamin A which are needed to produce malformations in vivo, since these are quite unrelated to normal physiological requirements and may affect the maternal organism as well as the embryos: Langman & Welch (1966) gave three injections of 30000 i.u.; Kochhar (1968) gave three injections of 60000 i.u.; Morriss (1972) gave a single injection of 100000 i.u. If vitamin A affects embryos directly, the amount actually reaching them may be only a small fraction of the amount given to the dam; determination of the effective concentrations in vitro would give an indication of the serum levels of active vitamin A in the in vivo situation.

**MATERIALS AND METHODS**

Rat 'egg-cylinders' were explanted during the afternoon of day 8 of pregnancy (day 0 being the day of the positive vaginal smear) and cultured in circulating or static medium by the technique of New (1971). Explantation was carried out in Tyrode saline and Reichert's membrane opened. Those embryos to be cultured in circulating medium were anchored to a piece of fabric by means of the ectoplacental cone and Reichert's membrane before being placed in the circulator.

No more than five embryos were cultured in each circulator or watchglass and embryos from different litters were divided between different treatments in order to minimize initial differences.

The nutrient medium was immediately centrifuged (i.e.) serum containing 50 μg/ml streptomycin, which has been shown to support good development of egg-cylinders in vitro (Steele, 1972; Steele & New, 1974). This was prepared from blood drawn from the dorsal aorta of an anaesthetized rat. The blood was immediately centrifuged and the clot (free of erythrocytes) removed. After recentrifugation the serum was decanted. All centrifugations were carried out at 2500 rev/min for 5 min. For most cultures the medium was prepared on the same day as the explantation.

In circulator cultures 6–8 ml of medium was circulated at 5 ml/min and equilibrated with a gas mixture containing 5% CO₂ in air. In watchglass cultures 1 ml medium was equilibrated with 40% O₂. Sterile procedure was observed throughout the culture period.

Control embryos were grown in i.c. serum to which 0.01 ml absolute alcohol/ml was added. Experimental embryos were grown in i.c. serum containing
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Table 1. Embryonic development after 48 h in culture

<table>
<thead>
<tr>
<th>Vitamin A (µg/ml)</th>
<th>No. of embryos</th>
<th>Average yolk-sac diameter</th>
<th>No. with heartbeat</th>
<th>No. with protruding axis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A-treated Controls*</td>
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<td>A-treated Controls</td>
<td>A-treated Controls</td>
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<tr>
<td>20</td>
<td>4</td>
<td>3</td>
<td>1.2</td>
<td>1.7</td>
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<tr>
<td>10</td>
<td>4</td>
<td>3</td>
<td>1.5</td>
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<td>5</td>
<td>3</td>
<td>3</td>
<td>1.6</td>
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<tr>
<td>3</td>
<td>16</td>
<td>20</td>
<td>1.6</td>
<td>1.7</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>9</td>
<td>1.65</td>
<td>1.8</td>
</tr>
<tr>
<td>0.5</td>
<td>32</td>
<td>28</td>
<td>1.7</td>
<td>1.8</td>
</tr>
</tbody>
</table>

* Where more than one experimental culture shared the same control culture, the controls appear more than once in this column. Total no. control embryos = 49.
† Other two 'embryos' indistinct.

Retinol (vitamin A alcohol) dissolved in absolute alcohol, in final concentrations ranging from 0.5–20.0 µg/ml; the volume added was 0.01 ml/ml of medium in all cultures.

The embryos were examined with a dissecting microscope after 24 and 48 h and the yolk-sac diameter, activity of the heart and general appearance recorded. Paraffin sections of some embryos were prepared for more detailed morphological investigation.

Some cultured embryos were prepared for electron microscopy. At 1 µg vitamin A/ml, embryos were examined after 1 h (8), 2 h (2) and 6 h (2) (numbers in brackets = embryos examined in each case). At 0.5 µg/ml embryos were examined after 10 min (2), 20 min (7), 30 min (2), 1 h (8), 2 h (7), 4 h (2) and 6 h (3). (These were all watchglass cultures, so that some embryos in the same dish could be left to continue developing to 48 h.) The embryos were rinsed in Tyrode saline, then fixed in 4% cacodylate-buffered glutaraldehyde, post-fixed in osmium tetroxide, and embedded in Araldite. Thin sections were double stained and viewed in a Philips EM 300.

RESULTS

Development achieved by embryos after culture for 48 hours (see Table 1)

Embryos cultured in medium containing 5, 10 or 20 µg/ml vitamin A developed very poorly. After 48 h none had a beating heart, and the embryo proper was represented only by a thickened area on the yolk-sac wall in which little or no differentiation was apparent when viewed with a dissecting microscope. There was a definite longitudinal axis, the posterior part of which formed a straight and rigid protrusion from the yolk sac. As in previous studies on culture techniques (Le Goascogne & Brun, 1969; Steele, 1972), this protrusion was occasionally observed in control embryos (7 out of 49 of all controls) but in these it was always smaller, less rigid, and therefore curved.
Figs. 1–4. Embryos cultured for 48 h, to show height of cephalic neural folds, amount and density of cephalic mesenchyme, and extent of foregut development.

Fig. 1. L.S. control embryo, 12 sections lateral to the mid-sagittal plane.

Fig. 2. L.S. embryo cultured in serum containing 0.5 μg vitamin A/ml, 12 sections lateral to the mid-sagittal plane. Flatter neural folds and less cephalic mesoderm than control; posterior part of axis protrudes from yolk sac.

Fig. 3. Oblique coronal plane, control embryo, position of greatest height of neural fold.

Fig. 4. Coronal plane, embryo cultured in serum containing 0.5 μg vitamin A/ml, showing flattened neural folds and great extension of foregut into head.

Embryos cultured in medium containing 3 μg/ml vitamin A developed more normally. Four out of 16 developed a heartbeat, although at 48 h the rate was only 35 beats/min compared with 60–80 beats/min in the controls.

Development was most similar to that of control embryos at a vitamin A concentration of 0.5 μg/ml; the protrusion of the posterior part of the axis was greatly reduced in extent, and occurred in only 9 out of 32 embryos. The development of the head differed from that of the controls in that the neural folds were flat and widely open. This was confirmed in sections (Figs. 1–4), which also showed (not illustrated) that the paraxial mesoderm was diffuse and had
**Figures 5-7**

**Fig. 5.** 0.5 μg vitamin A/ml, 10 min culture. Extra-embryonic endoderm: distorted nuclei with swollen nuclear membranes.

**Fig. 6.** 0.5 μg vitamin A/ml, 30 min culture. Embryonic ectoderm (with basement membrane) and mesoderm: 'buds' (b), vacuoles (arrowed) and condensed mitochondria.

**Fig. 7.** 0.5 μg vitamin A/ml, 30 min culture. Extra-embryonic endoderm: large lipid droplet (l), increased intercellular spacing.
not formed discrete somites. The closed area of the neural tube was never straight, but had multiple irregular lateral flexures. The foregut was deeper in experimental embryos at this concentration than in the controls.

Electron microscopy (Figs. 5–7)

In all embryos cultured for 30 min or more at vitamin A concentrations of 1 μg/ml and 0·5 μg/ml, all the cytological abnormalities previously observed in embryos treated in vivo with vitamin A were seen. These were: increase in the surface area of the plasma membrane and intracellular membranes; autophagic and/or heterophagic vacuoles; small vacuoles close to the cell surface; cell death; ‘budding’ of cells, particularly the basal surface of ectoderm cells; distorted ectodermal nuclei with enlarged intermembranous spaces; condensed mitochondria; wider spacing of cells with an increased volume of extracellular fluid; increase in the number of large lipid droplets of medium electron density. Except where stated, these effects occurred in the cells of all three germ layers. The embryos viewed after only 10 min of culture at 0·5 μg vitamin A/ml were similar except that the effects were apparent only in the endoderm, and the increase in spacing of these cells was slight; advanced cell degeneration and death was only apparent in the parietal endodermal (Reichert’s membrane) cells, which cannot be considered a functional part of the extra-embryonic membrane system in the culture situation.

In the controls, two of the above effects were constantly observed: from 30 min of culture onwards, all embryos contained some cells with autophagic or heterophagic vacuoles, and the mitochondria appeared to be condensed compared with those of embryos of the same age fixed immediately after explantation.

DISCUSSION

Allowing for differences directly attributable to development in vitro, the morphology of embryos cultured for 48 h in serum containing 0·5 μg vitamin A/ml is strikingly similar to that of embryos explanted 48 h after the maternal administration of 100 000 i.u. (60 000 μg) vitamin A (Morriss, 1972). This suggests that vitamin A affects embryonic development in vivo by direct action on the embryos. In both of these experimental situations the development of the cephalic neural folds was retarded; this was associated with reduction of cephalic mesenchyme, exaggeration of the cephalic flexure and greater extension of the foregut into the head than in the controls.

Further evidence for direct action was provided by the electron microscopic observations. At the lowest concentrations (1·0 and 0·5 μg/ml) all the cytological effects previously seen in vivo (Morriss, 1973a) were present in the cultured embryos within 30 min of the start of culture. Two phenomena (autophagic or heterophagic vacuoles and condensed mitochondria) were present in both
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experimental and control cultured embryos, and therefore cannot be ascribed to the action of vitamin A. In the previous in vivo study they were seen in embryos from vitamin A-treated but not control dams; since they had not been described in other electron microscopic studies on excess vitamin A, they were thought to be maternal-mediated effects (Morriss, 1973a,b).

At higher concentrations (5–20 μg vitamin A/ml serum) embryonic development was retarded far more than at 0.5 μg/ml, but in addition there was an almost complete failure of differentiation. Both of these effects may be attributable to a decrease in DNA synthesis, since this has been demonstrated in the presence of excess vitamin A in embryonic chick-limb cartilage (Dingle, Lucy & Fell, 1961) and in various tissues of 13-day rat embryos (Kochhar, 1968).

The results also indicate that young differentiating embryos are more sensitive to vitamin A excess than are differentiated tissues. Cultured pig articular cartilage is sensitive to minimum levels of 5 i.u. (3 μg vitamin A/ml medium) (Barratt, 1973) and embryonic chick epidermis to 2.5 i.u. (1.5 μg/ml) (Fell & Rinaldini, 1965). Embryos cultured at lower concentrations and for shorter exposure times would yield further information on this point.

Since such low concentrations affected in vitro development, it seems that only very small blood concentrations of active vitamin A were reaching the embryos affected in vivo. The great discrepancy between effective amounts in vivo and in vitro may be related to factors involved in maternal vitamin A storage, transport, and metabolism.

In contrast to vitamin A alcohol (retinol), retinyl esters have little or no action on tissues in vitro (Fell, Dingle & Webb, 1962; Roels, 1969). Preliminary work (Morriss & Steele, unpublished) indicated that this holds true for whole embryos under culture conditions similar to those of the present study. It thus appears likely that vitamin A affects embryos in vivo in the alcohol form. Vitamin A is stored in the liver as the ester (Moore, 1957). It is hydrolysed to retinol in the liver cells (Mahadevan, Ayyoub & Roels, 1966) and transported in the plasma attached to a lipoprotein, retinol-binding protein (RBP) (Raz, Kanai & Goodman, 1968). RBP is synthesized in the liver, and its secretion into the plasma is related to the availability of vitamin A (Goodman, Muto, Smith & Milch, 1972). Retinol-RBP may also be bound to a prealbumin; this complex releases retinol less easily than retinol-RBP alone, so may serve to stabilize the retinol in the bloodstream (Goodman, 1969). Dingle, Fell & Goodman (1972) found that when bound to RBP, retinol has no effect on cultured embryonic chick limb-bones, although it does have an effect when bound to non-specific serum proteins. Thus excess serum vitamin A should be defined in terms of the concentration of free and non-specifically bound retinol. The free fraction is not indicated in reported serum assays, although it is clearly critical for the adverse systemic as well as developmental effects.

The fact that very low levels of retinol affect embryonic development may be a significant factor in human teratogenesis. Normal human serum vitamin A
levels are 30–50 μg/100 ml (Russell, Bagheri & Boyer, 1973). High serum vitamin A levels due to decreased storage capacity have been associated with liver injuries such as cirrhosis and carbon tetrachloride poisoning (Baker & Frank, 1968). Chronic vitamin A intoxication is followed by high serum levels only when the liver capacity has been exceeded, but once this stage is reached serum levels may remain high for months or years after vitamin A ingestion has ceased (Munter, Perry & Ludwig, 1971). Large doses of vitamin A may result in extensive fibrosis, portal hypertension, and ascites (Russell et al. 1973), so that relatively small amounts of vitamin A bring about rapid increases in serum levels.

In human pregnancy, the maternal serum vitamin A determinations reported in the literature do not cover the period during which the developing embryo is most vulnerable to teratogens (e.g. Wild, Schorah & Smithells, 1974; Gal, Sharman & Pryse-Davies, 1972). Appropriate timing is difficult to achieve since the embryo is at the primitive streak stage at the time of the first missed period, and neural tube closure is complete 11 days later (Langman, 1969). However, congenitally malformed infants have been reported following actual ingestion of large amounts of vitamin A by women in the first month of pregnancy; in one case the urogenital system was affected (Pilotti & Scorta, 1965), in another, the heart and palate and in a third the palate alone (D. E. Poswillo, personal communication).

Clearly, even in the absence of exogenous sources of the vitamin, liver damage leading to raised serum vitamin A during early human pregnancy might raise the free retinol levels enough to cause congenital malformation. Whether this does in fact occur has yet to be established.

The authors wish to acknowledge the support of the Wellcome Trust and Medical Research Council. We are grateful to Professors R. J. Harrison and C. R. Austin for the provision of laboratory facilities, to Mr G. Owen for photography, to Dr A. D. Thomson for providing the clinical references, and to Dame Honor Fell, Professor C. R. Austin, Dr J. Herbert and Professor D. E. Poswillo for valuable criticism of the manuscript. The vitamin A was supplied by Dr N. T. Pollitt of Roche Products Ltd.

REFERENCES


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(Received 9 March 1974)