Susceptible stages and abnormal morphogenesis in the developing mouse limb, analysed in organ culture after transplacental exposure to vitamin A (retinoic acid)

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SUMMARY

The development of form and morphology of cartilage in limbs from control and retinoic acid-treated DBA/2J mice were studied in organ culture. Somite number at explantation (SE) ranged from 29 to 56. All cartilage segments except digits developed in cultures of 29-somite control limb-buds; digits developed well only from limb-buds of SE 45 and older stages. Maternal treatment with retinoic acid specifically inhibited the growth of limbs rather than that of the whole embryo; the percentage reduction in limb length in culture ranged between 20 and 40%, depending on the length of in utero exposure. The teratogenic effects of retinoic acid were expressed in culture even if the limb was explanted after only 1 h of in utero exposure. However, for a complete expression of the teratogenic effects, resulting in phocomelia, 24 h exposure was necessary. Limb-buds of 29 to 32-somite embryos were only mildly responsive to the teratogen. Embryos with 33–38 somites demonstrated the full extent of teratogenic effects. Embryos with more than 40 somites at the time of first exposure to retinoic acid escaped severe limb malformations except digital defects. Correlation between the developmental stage of the limb at the time of treatment and the final limb defect is attempted.

INTRODUCTION

Two approaches have exploited developmental variants in order to identify mechanisms in susceptible tissues which result in congenital malformations. Study of limbs of mutant embryos such as nanomelia in the fowl (Landauer, 1965; Mathews, 1967; Palmoski & Goetinck, 1972), talpid fowl (Hinchliffe & Ede, 1967), creeper chick (Elmer, 1968), hemimelia in the mouse (Green, 1967), luxoid mouse (Burd & Center, 1969), achondroplasia in the rabbit (Shepard, Fry & Moffett, 1969) have been productive. The alternate approach, involving experimentally induced limb deformity, has been exploited less often in the...
mammalian embryo because the intervening maternal system and intra-litter variation result in lack of predictability for well controlled embryo manipulation.

Retinoic acid (vitamin A acid), when administered to pregnant mice of DBA/2J strain on the 12th day, resulted in 100% of fetuses with skeletal malformations of the extremities (Fig. 1). The type and frequency of malformations were modified by time of treatment (Kochhar, 1973). The variability observed within litters was presumed to reflect undetectable deviations in developmental age among litter-mates at the time of teratogenic treatment.

*In vitro* development subsequent to induction of limb deformity has provided information about the precise stages at the time of treatment and has enabled assessment of developmental capability of treated mouse limb-buds. The present investigation using retinoic acid had the objectives: (1) to identify precisely the initial susceptible stage of the limb-bud, (2) to correlate exact developmental stages with the final teratogenic response, and (3) to measure minimal and optimal transplacental exposure periods resulting in limb teratogenesis.
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MATERIALS AND METHODS

Animals
Timed-pregnant mice of inbred strain DBA/2J were obtained from the Jackson Laboratory, Bar Harbor, Maine during the first week of gestation. The females were considered to be in the first day of pregnancy on the day the vaginal plug was discovered. They were provided with food pellets (Purina) and water ad libitum and housed in modules maintained at constant temperature provided with 12 h light and dark cycles.

Induction of limb deformity
The animals were divided into two groups. One group served as control while the animals in the other group received retinoic acid. All trans retinoic acid (Sigma) was suspended in cottonseed oil to give a concentration of 20 mg/ml. At 10 a.m. on the 12th or 13th day of pregnancy each animal in the treated group received 80 mg/kg retinoic acid by oral intubation. The animals were slightly etherized and killed by cervical dislocation at 1 to 24 h after treatment. Control animals were either untreated or treated with the solvent carrier, cottonseed oil.

Organ culture procedure
Both uterine horns from each female were removed to a dish of sterile Tyrode saline; each embryo was stripped of its membranes, and the number of somite pairs counted under the dissecting microscope. With sharpened cataract knives the fore- and hindlimb-buds were dissected away from the body along a line immediately lateral to the row of somites. Fore- and hindlimbs of each side were transferred to a small piece of ultra-thin Millipore filter (25 μm thickness, 0.45 μm pore size) which was introduced into the culture chamber assembly (Fig. 2). The filter carrying the explants was supported by a grid of expanded stainless steel. The nutrient medium consisted of 75% BGJ medium (Biggers, Gwatkin & Heyner, 1961; obtained from Difco) and 25% fetal calf serum (FCS) (Flow Laboratories) and supplemented with 150 μg/ml ascorbic acid, 12.5 μg/ml streptomycin, and 7.5 μg/ml penicillin G. The level of the medium in the culture dish was adjusted so that it bathed only the underside of the filter and limb-buds were not submerged. The cultures were incubated at 38 °C in an atmosphere of 5% CO₂ in humidified air. Medium was replaced every 3 days.

The maximum culture period used in these experiments was 9 days (day of culture = 0 day). More commonly, cultures were terminated at 24 h, 48 h, 72 h and 5–6 days after explantation. The cultured limbs, still attached to the filters on which they were grown, were rinsed briefly in saline, fixed in Bouin's solution for 1 day, and stained either with aqueous 1% alcian blue in 3% acetic acid or with 0.1% toluidine blue in 70% ethanol. After dehydration the
explants were briefly immersed in acetone to partially solubilize the opaque material of the filter, to aid photography of the whole mounts. The cultures were cleared and stored in cedarwood oil.

Lengths of limbs, uncultured (0 day) or cultured, were measured as cleared whole mounts by means of a micrometer in the eye-piece of a stereomicroscope. Since the limb developed flexures in vitro just as in vivo (see Results), the lengths are reported as aggregates of separate measurements on each segment of the limb.

RESULTS

Effect of retinoic acid on growth of embryo – rate of somite formation (Table 1)

Embryos at 10 a.m. on the 12th day possessed 29–41 pairs of somites, an average of 35. Treatment of the mother at this time with retinoic acid resulted in an average of 45 somite pairs after 24 h. Control embryos had an average of 46 somite pairs. Therefore, between the 12th and 13th day of gestation about one somite pair was added every 2 h. Retinoic acid administered at 10 a.m. on the 13th day resulted in an average of 52 somite pairs after a 24 h period; controls had an average of 56 somite pairs. Table 1 indicates that 24 h after a teratogenic dose of retinoic acid the number of somite pairs, hence growth of the embryo, was similar to controls.

Effect of retinoic acid on growth in length of limb-buds (Table 2)

The forelimb of 37-somite control embryos measured 1.1 mm in length. Table 2 indicates that although limb-buds from embryos younger than 45 somites failed to give a detectable response after 24 h of exposure to retinoic acid, those from older embryos showed a definite reduction in length, compared with controls. On the 18th day of gestation, embryos that had been treated with retinoic acid 6 days previously had limbs 25% shorter than those of controls (Table 2).
Limb development in mouse embryos

Table 1. Effect of transplacental treatment with retinoic acid for 24 h on growth of embryos, i.e. on number of somites

<table>
<thead>
<tr>
<th>Day observed</th>
<th>Number of embryos</th>
<th>Number of somite pairs (average)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated*</td>
</tr>
<tr>
<td>12th, 10 a.m.</td>
<td>134</td>
<td>—</td>
</tr>
<tr>
<td>13th, 10 a.m.</td>
<td>38</td>
<td>45</td>
</tr>
<tr>
<td>14th, 10 a.m.</td>
<td>37</td>
<td>35</td>
</tr>
</tbody>
</table>

* Eighty mg/kg retinoic acid administration orally 24 h previously.

Table 2. Effect of transplacental treatment with retinoic acid 24 h or 6 days previously on growth in length (mm)* of forelimbs

<table>
<thead>
<tr>
<th>Developmental age at time of observation</th>
<th>Control</th>
<th>Treated†</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 somites</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>43 somites</td>
<td>1.9</td>
<td>2.0</td>
</tr>
<tr>
<td>45 somites</td>
<td>2.4</td>
<td>2.0</td>
</tr>
<tr>
<td>47 somites</td>
<td>2.9</td>
<td>2.3</td>
</tr>
<tr>
<td>50 somites</td>
<td>3.8</td>
<td>3.0</td>
</tr>
<tr>
<td>18th day</td>
<td>9.7</td>
<td>7.3</td>
</tr>
</tbody>
</table>

* Average of at least five limbs.
† Eighty mg/kg retinoic acid administered orally 24 h or 6 days previously.

Growth and development of limb-buds in culture

General remarks

Within 24 h explanted limb-buds firmly attached themselves to the filter, mostly through cell growth from the proximal cut end of the bud; the distal end remained free initially. The area of attachment continued to expand and considerable increase in length was noticed within 3 days. By 5–6 days a consistent pattern of limb development was obtained. Proximo-distal orientation was recognized by the girdle on one end and the distinct, epithelial covered autopod on the other. Irrespective of developmental age of the explanted limbs, distortion resulted from attachment to the filter. Since the hindlimbs always showed more distortion than the forelimb, the following description is limited exclusively to the forelimb. Foci of necrosis were observed in the cultured limbs only if they were derived from embryos having more than 47 somites. Cartilage was the predominant tissue in 5- to 6-day cultures, although some muscle fibers were observed. Further myogenic areas appeared in 9-day cultures, but cartilage still dominated the limb.
Donor embryos, ranging in SE stages from 30-54, were obtained from control mothers (•), and from mothers which had been treated with retinoic acid 6 h (△) or 24 h (○) before sacrifice. Each point is an average of at least five readings.

Control limb-buds in 5- to 6-day cultures

The development and growth attained by limbs in culture depended on the initial developmental age of the donor embryos; the age was denoted by the number of somite pairs present at the time of explantation (SE, Fig. 3). The forelimb-bud of a 37-somite embryo grew from an average initial length of 1.1 mm to an average of 2.6 mm after a period of 6 days in culture. A similar 2- to 3-fold increase in length was obtained in 5- to 6-day cultures from control embryos of SE stages 29–54 (Fig. 3).

The scapula developed as a triangular cartilage with a prominent acromion process in all cultured limb-buds. At SE 29 stage the humerus was most prominent as a straight bar of cartilage (Fig. 4). In SE 35 and older stages, ulna and radius also became prominent. Adequate development of the digits was obtained only in SE 45 and older stages; in younger embryos the cartilages of individual digits were not always separately recognized in 5- to 6-day cultures.

The forelimb in culture developed two characteristic flexures. One, observed in limb-buds of all embryonic stages, occurred between humerus and radius–ulna and could be considered the site of the future elbow joint (Fig. 4). The concave side of this flexure was always towards the radial aspect. The second flexure was observed only in limb-buds of SE 35 stage and older; it occurred...
within the shaft of the humeral cartilage, just distal to its joint with the scapula. The direction of this flexure was approximately 90° away from the first flexure. The radius showed a flexure of its own in cultured limb-buds of SE 35 and older stages.

**Treated limb-buds in 5- to 6-day cultures**

The inhibitory effect of the teratogenic treatment on limb growth was apparent when these limbs were cultured. Limbs cultured after an *in utero* teratogenic exposure of 6 h, those from embryos of SE stages 37–39, showed a 20% reduction in length compared to controls (Fig. 3). The reduction was in the range of 40% if limbs were cultured after a 24 h exposure; the effect was most pronounced in embryos of SE stages 46–50 (Fig. 3).

Limb-buds explanted even 1 h after maternal treatment with retinoic acid demonstrated some overt atypical development in culture. The degree of this deviation in development was, however, found to be variable. This variability was observed in limb-buds cultured at each of the subsequent time intervals following treatment; however, the severity of abnormality increased with increasing period of *in utero* exposure. Most severe malformations, which could be described as phocomelia, were produced if the limb-buds were cultured after a full 24 h *in utero* exposure.
Fig. 5 shows a series of limbs from embryos with increasing SE stages cultured after 6 h in utero exposure. From the shapes of cartilaginous segments and the extent of development obtained after the 6-day culture period, it appeared as if the cultures were made from much younger limb-buds than the actual SE stage. The scapula formed almost normally. The other cartilage segments such as humerus, radius and ulna were shorter and more slender than controls (Fig. 5; compare with Fig. 4). The outline of the autopod was deformed; it was more flattened than normal on the radial (preaxial) side.

Fig. 6 shows a series of limbs cultured for 5–6 days subsequent to a 24 h in utero exposure to retinoic acid. The range of SE was 41–54. At both extremes of this developmental period the limb showed less abnormal development than in the intervening duration. When explanted at SE stage 41–42 (first exposure when embryo was at 29–30 somite stage, computed at the rate of 1 somite pair added every 2 h; Table 1), all cartilage segments made their appearance but they were reduced in size. The whole limb was smaller than a control limb cultured at SE 41 stage, but was similar in size to one explanted at SE 35–37 (Fig. 6; compare with Fig. 4). The autopod was pointed rather than expanded. Severe malformations were observed when explanted at SE stages 45–49. The humerus was completely missing, radius-ulna was present either as a single V-shaped cartilage (SE 45) or as separate, variably reduced nodules of cartilage (SE 46, 47). At SE 49–50, humerus–radius–ulna were present as a single Y-shaped cartilage which did not show sharp borders in alcian blue-stained whole
mounts. The autopod was pointed and severely deformed. When explanted at SE 52–54, the limb showed less severe abnormalities than at earlier stages. Almost normal growth was obtained from limbs explanted at SE 54 (first exposure when the embryo was at 40–42 somite stage). However, the autopod showed three or four digits instead of the normal five (Fig. 6).

Comparison of control and treated limb-buds in 0–3 day cultures

Fig. 7 shows an uncultured (0 day) limb of a 37-somite control embryo and 2-day limb cultures made from a series of SE stages. At SE stages 35–38 the limb showed only diffuse alcian blue-staining and no toluidine blue-metachromasia in 0-day or 1-day cultures. In 2-day cultures overt chondrogenesis was first observed in the scapular and humeral segments; there were no sharp borders to these chondrogenic areas. Diffuse alcian blue-staining was observed in distal areas. Cultures of increasing SE stages showed advanced chondrogenesis even after 2 days of culture. At SE 45–46, 2-day cultures showed advanced chondrogenesis in all segments including digits (Fig. 7).

Comparing the control series with cultures of treated limbs explanted at 6 and 24 h after in utero exposure, a delay in the onset of chondrogenesis was
observed in the treated series (Fig. 8). Growth inhibition was not as apparent or as extensive in the 6 h series as it was in the 24 h series. In the latter, an SE 41–42 stage 2-day culture was comparable to a control SE 35 stage culture (Fig. 8). When control and 24 h treated limbs were compared through the 0- to 3-day culture period (Fig. 9, SE 47–49), all aspects of development were abnormal. The autopod failed to expand in the treated limb, chondrogenesis lagged behind the control, and all cartilage segments were small or missing. With further culture to 6 days, treated limbs showed extensive chondrogenesis, but the cartilages were grossly distorted in shape and their borders were not as well defined as in the controls (Fig. 9).

DISCUSSION

The development of vertebrate limbs in avascular culture has previously met with only limited success (Searls, 1968; Shepard & Bass, 1970; Yasuda, 1973). This report extends our previous observations (Aydelotte & Kochhar, 1972) on the culture of mouse limb-buds in a partially defined medium. A consistent pattern of limb development was obtained, dependent only on the developmental age of the donor embryo. The youngest limb-bud cultured in this study, at SE stage 29, corresponds in development to stage 19 chick embryo (Hamburger & Hamilton, 1951). Such a limb-bud in culture developed all cartilage segments of the limb except the digits. After a 6-day culture period limbs from all stages showed a 2- to 3-fold increase in length. All cartilage
Table 3. Incidence of abnormal limb development in culture as a function of developmental age (number of somites) at the time of retinoic acid treatment*

<table>
<thead>
<tr>
<th>Number of somites at time of treatment</th>
<th>Number of limbs cultured</th>
<th>Limbs affected No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>29-32</td>
<td>24</td>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td>33-36</td>
<td>24</td>
<td>23</td>
<td>96</td>
</tr>
<tr>
<td>37-40</td>
<td>42</td>
<td>37</td>
<td>88</td>
</tr>
<tr>
<td>41-44</td>
<td>16</td>
<td>4</td>
<td>25</td>
</tr>
</tbody>
</table>

* Eighty mg/kg retinoic acid administered orally.

segments increased in length including the radius-ulna. Good development of digits was consistently obtained in limbs cultured at SE 45 or older.

The forelimb that develops in situ shows two intrinsic flexures, one at the shoulder joint and the other at the elbow joint (Fig. 1). Both of these flexures are built into the limb-buds of SE 35 stage and older, although the proximal flexure which developed in culture occurred in the humeral shaft rather than between it and the scapula (Fig. 4).

It was shown in a previous study (Kochhar, 1973) that the incidence of forelimb malformations was 100% in fetuses from animals treated with retinoic acid at 10 a.m. on the 12th gestational day. The data from this study confirmed that observation and further revealed that the incidence of abnormal development in culture ranged between 88 and 100% in embryos which had 29-40 somites at the time of their first exposure to retinoic acid (Table 3). This incidence declined to 25% if the embryos had 41-44 somites at the time of treatment (Table 3). The degree of abnormality shown by each limb depended on the length of its in utero exposure to retinoic acid.

No change in the rate of somite formation was found as a result of teratogenic treatment on the 12th day (Table 1). This fact provided validity to our use of the somite number as an indicator of developmental age when comparing limb development in control and treated embryos.

Limbs of treated embryos demonstrated a considerable retardation of growth in length when compared to control embryos (Table 2). This indicated that although general embryonic development in treated embryos continued at a normal rate, that of the limbs was specifically depressed. The inhibitory effect of the teratogenic treatment on the limb growth became much more pronounced when such limbs were cultured. Limbs cultured after an in utero teratogenic exposure of 6 h showed a 20% reduction in length compared to the control value whereas the reduction was in the range of 40% if limbs were cultured after a 24 h teratogenic exposure (Fig. 3). No growth-inhibitory effect of the teratogen was noticeable if SE stage was 54.

The data in Table 1 permitted us to calculate from the SE stage the actual
somite stage of the embryo at the time of its first in utero exposure to retinoic acid. This determination was considered essential for our further efforts to identify cellular and developmental phenomena in the limb which provide targets for teratogenesis. Based on this calculation, i.e. from estimations that 1 somite pair was added every 2 h, we found that limb-buds from 29–32 somite embryos responded by showing only a mild teratogenic effect (Figs. 5, 6). Embryos with 33–38 somites demonstrated the full extent of teratogenic effect (Fig. 6), with 35 somite embryos showing almost the complete absence of humerus, radius and ulna, described previously as phocomelia (Kochhar, 1973). Embryos with more than 40 somites escaped severe limb malformations except for having ectrodactyly (Fig. 6).

Besides an effect on growth, the treated limbs also showed an inhibitory effect on chondrogenesis. A delay in the initiation of chondrogenesis was most evident in all segments of the limbs cultured from embryos of SE stages 35–38 (compare Figs. 7 and 8). In older stages scapular chondrogenesis showed no apparent difference from controls but chondrogenesis in all other areas was delayed (Fig. 9). The cartilage which appeared in treated limbs in 6-day cultures was distorted in form and showed a slight alteration in alcian blue-stained whole mounts (Fig. 9). Under the light microscope the intercellular matrix of this cartilage showed no structural alterations from controls, although the chondrocytes possessed smoother outlines than the usually scalloped appearance of control chondrocytes (Kochhar, unpublished observations). Examination of the cells and the matrix with the electron microscope is in progress. Definite alterations in the physiology and structure of the cells and matrix are predicted because of the fact that large areas of the treated cartilage were not replaced by bone as they normally were in the control fetus (Fig. 1).

Profound changes were observed in the hand region (autopod) of the treated cultures; it failed to attain the broad, paddle-shaped appearance of the control cultures. The preaxial aspect of the autopod was more affected than the postaxial, resulting in preaxial ectrodactyly (Figs. 5–9). More often, however, chondrogenic areas in the autopod did not organize into separate digits (Fig. 9).

Our results indicated that the teratogenic effects of in utero exposure of the limb to retinoic acid continued to be observed after these limbs were explanted in a culture medium having no excess of vitamin A. Additional studies have shown that similar defects were produced if previously untreated limbs were cultured in a medium containing an excess of vitamin A (Kochhar & Aydelotte, unpublished observations). Such evidence therefore permits us to propose that the teratogenic effects of vitamin A compounds may be the outcome of their direct influence on the embryo itself rather than on the maternal or placental systems, as has been reported (Geelen, 1972).

Janners & Searls (1970) have reported that in the chick at stage 19 the proliferative index in all regions of the limb-bud was 100 %. This stage corresponds to the 29-somite stage in the mouse, a stage when retinoic acid produced only
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a mild teratogenic effect. Also, it is known that the limb-bud of younger embryos did not respond at all to the teratogen since no malformations were produced by treatment on the 11th day of gestation (Kochhar, 1973). This would indicate that retinoic acid had no influence on those factors operating in the initiation and early development of the limb in the mouse embryo (Searls & Janners, 1971). It was in embryos of older stages, corresponding in age to stage 23 of the chick embryo, that the limb began to show maximal teratogenic effects. It is at this stage that the proliferative index in the prospective chondrogenic area declines to 25% and an increase in the rate of glycosaminoglycan synthesis is noticed (Janners & Searls, 1970; Searls, 1965a, b). In further studies we hope to focus on this stage to discover correlations between cell proliferation and differentiation of cartilage on the one hand and the teratogenic effects on the other.

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REFERENCES


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