Skeletal malformations in the Holtzma rat embryo following the administration of thalidomide

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WITH TWO PLATES

INTRODUCTION

Since the occurrence of the thalidomide tragedy in man, fairly extensive animal research has been done on the rat (King & Kendrick, 1962; Bignami et al., 1962; Klein Obbink & Dalderup, 1963; McColl et al., 1963; Moore et al., 1964), mouse (Giroud et al., 1962; Woollam, 1962; DiPaolo, 1963), rabbit (Felisati, 1962; Somers, 1962; Spencer, 1962), monkey (Lucey & Behrman, 1963), chicken (Kemper, 1962; Yang et al., 1963; Rickenbacher, 1964) and Drosophila (Lüers, 1962; Parkash, 1962).

There are, however, only a few instances where fetal deformities have resulted from the administration of thalidomide to rats. King & Kendrick (1962) gave thalidomide orally and intraperitoneally to Sprague-Dawley rats in doses ranging from 10 to 50 mg. and observed the following gross malformations in 6.9 per cent of the fetuses: hamartoma of the palate with accessory incisors, malrotation of the hind limbs and absence of the tail. Alizarin staining of the skeletons revealed forty additional anomalies raising the total percentage to 14.6. In the Wistar SM rat, Bignami et al. (1962) noted an increase in the number of resorbed implantations and a decrease in the average weight of fetuses when 200 and 500 mg./kg. of thalidomide were administered orally. Abnormalities and malposition of the limbs and tails were also observed in two liveborn pups. McColl et al. (1963) detected branched and fused ribs, 'scrambled' sternebrae and double vertebral centra in alizarin-stained rat fetuses whose mothers had been fed 1 to 2 per cent thalidomide in their diet. Klein Obbink & Dalderup (1963) noted an increased frequency of abnormal ossification of the centres of the 5th sternebrae in Wistar rats. Although this defect was occasionally seen in control fetuses, they concluded that the higher the dose of thalidomide, the higher was the incidence and seriousness of deformities of the 5th sternebral centre. Rarely centres of the 2nd and 4th sternebrae were affected. In a later study Klein Obbink & Dalderup

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(1964) concluded that thalidomide induced anomalies, however, they pointed out that there seemed to be fairly wide variations of normal development which were not related to the drug. They found that bones which showed considerable variation in control fetuses were also the ones most susceptible to the effects of thalidomide.

The present study is a further investigation of the effects of thalidomide on rat development. It is hoped that from such studies, information will accumulate that will be helpful in designing more reliable teratogenicity tests of drugs in animals.

MATERIALS AND METHODS

Holtzman albino rats (descended from the Sprague-Dawley strain) were used in this study. Virgin rats (200 to 260 g.) were formed into groups of ten animals for control and treatment by randomizing. The rats were ear-marked and then groups of four females were placed with a male in breeding cages. The day on which spermatozoa were observed in vaginal smears was considered as day 0 of gestation. Mated females were placed in separate cages to await treatment with thalidomide and/or sodium carboxymethylcellulose (CMC).

The thalidomide was suspended in a 1 per cent. solution of CMC, medium viscosity. Table 1 shows the animal groups, doses, methods and days of administration used.

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<th>Method of administration</th>
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Key to Symbols: e.g. 1AT
1—refers to the dosage given to the group, i.e. 25 mg.
A—refers to the intraperitoneal method of administration. B, as in 1BT, refers to the stomach-tube method of administration.
T—indicates a treated group. Control groups are referred to in the text as C. Therefore 1AC is a control group that received CMC by intraperitoneal injection.

All groups contained ten animals. Control groups (C) were given CMC on the same days of gestation and by the same methods; the amount given (0·2, 0·3 or 1·5 c.c.) was the same as the volume used to suspend the drug for treated animals,
e.g., group 1AC received 0·2 c.c. of CMC, the amount used to suspend the thalidomide in the treated group 1AT.

The apparatus used for tubal administration of the drug was made up as follows (Plate 1, Fig. A): a three-way Luer-Lok stopcock was attached to a 1 or 5 c.c. syringe, depending on the amount to be administered; a blunted 18G Luer-Lok needle was attached to the stopcock and a 2½-in. piece of fine polyethylene tubing was fitted over the needle. The same apparatus, excluding the stopcock, was used for administration of CMC to the control groups. Thalidomide was administered intraperitoneally (IP) to the 25 and 50 mg. series using a 1 c.c. tuberculin syringe and a 1-in. 21G disposable needle. In the 500 mg. group a 5 c.c. syringe was used with an 18G needle.

The rats were killed on the 20th day of gestation, using ether or chloroform anesthesia, and the abdominal viscera were exposed by a mid-line abdominal incision. The uterine horns were removed and carefully examined for macerated fetuses and resorption sites. Fetuses were removed from the horns, counted, weighed and measured. After evisceration the fetuses were fixed separately in jars of 95 per cent. alcohol for at least 1 week. The placentas were measured and the average size recorded. Placentas, macerated fetuses and resorption sites, if present, were fixed and stored in modified Davidson's solution (Moore & Barr, 1954).

Following fixation fetuses were placed in (a) acetone to dissolve the body fats, (b) 1 per cent. potassium hydroxide to clear the skeletons, and (c) alizarin red S to stain the bones (Dawson's method; Gurr, 1962). After staining, the fetuses were examined under a dissecting microscope for skeletal abnormalities.

RESULTS

Fetuses in some treated litters were considerably smaller than most of those in control litters, but the average weight and length of the treated fetuses were not significantly different from the controls. The average number of fetuses in control groups was 10·8 and the average number in treated groups was 10·6.

There were more resorptions in treated groups; 53·8 per cent. of eighty mothers, compared with 36·6 per cent. of sixty control mothers. The chi-square test was used to calculate the probability of thalidomide causing resorptions and it was found to be 3·365 which is insignificant at the 5 per cent. level. However, if the 2·5 per cent. limit of significance is used, the magnitude of this difference is significant. At this level it seems justifiable to conclude that thalidomide tended to increase the frequency of resorptions in these animals.

Placental examinations revealed no significant differences between treated and control groups. Fused placentas were seen in two instances; once in a treated mother and once in a control female.

The total percentage of all treated and control fetuses showing clearly defined abnormalities is shown in Table 2.
In most instances abnormal limbs were not present. In one instance, however, a fetus had severely malformed limbs (Plate 1, Fig. B); its mother had received 25 mg. of thalidomide daily from the 10th to the 15th days of gestation.

Usually the rib abnormalities were supernumerary ribs. In one instance a fetus of the 50-mg. IP group had only eleven pairs of ribs (the normal is thirteen). However, in one control litter that had received 1-5 c.c. of CMC, there were seven fetuses which had either twelve pairs of ribs, or twelve ribs on one side and thirteen on the other. In such cases the 13th rib was extremely small (Plate 1, Fig. C). The accessory ribs were in the cervical and/or lumbar regions and sometimes they were paired. Although both treated and control fetuses had accessory ribs, the 500-mg. IP group showed nineteen fetuses with this anomaly (Table 3). One of these fetuses had fifteen ribs on one side (Plate 2, Fig. D).

About 30 min. after the administration of the 500-mg. dose of thalidomide the pregnant animals showed striking sedative effects, however they did not go to sleep. The untreated animals were more responsive to handling and probing than were these treated mothers.

**Results of Experiment I**

Each treated animal in this experiment (Table 1) received 25 mg. of thalidomide daily from days 10-15 of gestation by IP injection (1AT), or by stomach tube.

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**PLATE 1**

**FIG. A.** An illustration of the method of administering thalidomide by stomach tube. A three-way Luer-Lok stopcock is attached to a 1 c.c. tuberculin syringe. A blunted 18G Luer-Lok needle is attached to the stopcock and a $\frac{1}{2}$ in. piece of fine polyethylene tubing is fitted over the needle. The tube passes into the rat's stomach. The picture also illustrates the method of holding the animal for insertion of the tube.

**FIG. B.** The hind limbs of fetuses, 20 days, whose mother received 25 mg. of thalidomide intraperitoneally daily from the 10th to the 15th day of gestation. The limbs of the fetus on the left are normal, whereas those of the one on the right are abnormal. The right limb hangs straight down and shows little toe development. The left limb is phocomelic and resembles those observed in human and rabbit fetuses.

**FIG. C.** An alizarin-stained fetus, 20 days. This fetus' mother received 1-5 c.c. of sodium carboxymethylcellulose (CMC) intraperitoneally on the 8th day of gestation. It has twelve ribs on one side and thirteen on the other. The accessory rib, indicated by the arrow, is extremely small.
PLATE 1

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TEXT-FIG. 1. Percentage of abnormal vertebral centra in all treated and control litters.

Key to Text-Figs. 1–4
1A—25 mg. IP, days 10–15. 1B—25 mg. ST, days 10–15. 2A—50 mg. IP, days 8–12. 2B—50 mg. ST, days 8–12. 3A—25 mg. IP, days 8–12. 3B—25 mg. ST, days 8–12. 4A—500 mg. IP, day 8. 4B—500 mg. ST, day 8.

(1BT). Each control animal received 0.2 c.c. of CMC on the same days, either by IP injection (1AC), or by stomach tube (1BC).

Some fetuses of treated mothers exhibited the following abnormalities: abnormal vertebral centra (Text-fig. 1); abnormal vertebrae (Text-fig. 2); absence of the 5th sternebra (Text-fig. 3); and miscellaneous abnormalities.

TEXT-FIG. 2. Percentage of abnormal vertebrae, other than abnormalities of the centra, in treated fetuses.
TABLE 3

Various comparisons of control and treated fetuses

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<th>Days of Gestation</th>
<th>Living fetuses/group</th>
<th>Resorptions/group</th>
<th>5th sternebrae/group</th>
<th>Other sternebrae affected/group</th>
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* IP—intraperitoneally;  † ST—stomach tube. Each group consisted of ten animals.
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(Text-fig. 4) such as poor ossification of the pelvis or absence of the tail. There was doubt about the defects of toes and feet referred to in Table 3, as will be mentioned in the Discussion.

In the group injected intraperitoneally (1AT), there were 111 fetuses. Of these, 11-7 per cent. showed abnormal vertebral centra. The centra were usually ‘dumb-bell’ in shape, although sometimes they consisted of two parts. In some instances there was fusion of adjacent centra. In fifteen fetuses (13-5 per cent.) no 5th sternebra was visible.

Of 101 fetuses in the control group (1AC), 7-9 per cent. showed absence of the 5th sternebra. No other defects were observed.

There were ninety-one fetuses in the group given the drug by stomach tube (1BT): twenty-six (28-6 per cent.) showed abnormal vertebral centra; 12-1 per cent. had no ossification of vertebrae beyond the ilium; thirty-five fetuses (38-5 per cent.) showed no ossification of the 5th sternebra; twenty-four (26-4 per cent.) miscellaneous defects were also observed, such as retarded or lack of ossification of some or all bones of the pelvis.

There were 107 control fetuses (1BC), comparable to the treated group just described. Only five (4-7 per cent.) showed abnormal vertebral centra and 10-3 per cent. showed no ossification of the 5th sternebra.

Results of Experiment II

Each animal in this experiment (Table 1) received 50 mg. of thalidomide daily from days 8–12 of gestation by IP injection (2AT), or stomach tube (2BT). Each control animal received 0-3 c.c. of CMC on the same days by IP injection (2AC) or by stomach tube (2BC).

The most severe abnormalities that we have seen have been in fetuses whose mothers received the 50-mg. dose. The abnormalities are shown graphically in Text-figs. 1–4 (R2A and R2B groups). Other effects are recorded in Table 3.

In the intraperitoneally injected groups (2AT) there were 117 fetuses. Forty-six (39-3 per cent.) showed abnormal centra. Usually the centra were ‘dumb-bell-shaped’ or duplicated. In one instance the lumbo-sacral vertebrae were missing, along with two pairs of lower ribs (Plate 2, Fig. E). In two other fetuses, fusion of the vertebral centra resulted in a ladder-like appearance (Plate 2, Fig. F). In addition to the fetus which showed an absence of lumbo-sacral vertebrae, there were six other fetuses with poor ossification of sacral vertebrae; an overall percentage of 6 per cent. There were forty-four fetuses (37-6 per cent.) with no 5th sternebra; the sternebrae in two of these were also ‘scrambled’ (Plate 2, Fig. G). Some fetuses (2-6 per cent.) also had miscellaneous abnormalities.

In the controls (2AC) there were 124 young. Four fetuses (2-3 per cent.) had abnormal vertebral centra and 7-3 per cent. had no 5th sternal centres.

The group given the drug by stomach tube (2BT), contained 113 fetuses. Of these, 29-2 per cent. showed defects of the centra. In one fetus (0-9 per cent.) the
TEXT-FIG. 3. Percentage of all treated and control litters showing absence of the 5th sternebra.

vertebral column terminated at the ischium and no tail was present. Forty-four (38.9 per cent.) showed no ossification of the 5th sternebra; one fetus also had 'scrambled' sternebrae.

TEXT-FIG. 4. Percentage of miscellaneous abnormalities in all treated fetuses.
There were ninety-four fetuses in the stomach tube control group (2BC). Only 1.1 per cent. had no 5th sternal centre. No other anomalies were observed.

**Results of Experiment III**

Each animal in this group (Table 1) received 25 mg. of thalidomide daily from days 8–12 of gestation by IP injection (3AT), or by stomach tube (3BT) in 0.3 c.c. of CMC. The control groups (2AC and 2BC), described previously, were also used as controls for this experiment because the dosage given to the treated animals was the only alteration in the procedure. The major abnormalities are shown in the text-figures; other effects are recorded in Table 3.

There were 105 fetuses in the intraperitoneally-injected group (3AT). Twenty-seven (25.7 per cent.) had abnormal vertebral centra and twenty-eight fetuses (26.7 per cent.) showed no ossification of the 5th sternebra.

The stomach-tube group (3BT) produced ninety fetuses; 21.1 per cent. had abnormal vertebral centra. There was no 5th sternebra visible in 15.6 per cent. of the fetuses.

**Results of Experiment IV**

Each animal in this group (Table 1) received 500 mg. of thalidomide on day 8 of gestation by IP injection (4AT), or by stomach tube (4BT). Controls received 1.5 c.c. of CMC by the same routes on the same day.

The largest number of abnormal vertebral centra was observed in the IP group (Text-fig. 1). Fetuses in this group also showed the highest incidence of supernumerary ribs (Table 3). Other effects are also shown in this table. The IP-injected group produced 121 fetuses; 47.9 per cent. had abnormal vertebral centra.

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**Plate 2**

**Fig. D.** An alizarin-stained fetus, 20 days, with fifteen ribs on the right side. Two small accessory ribs are indicated by the arrows. This fetus' mother received 500 mg. of thalidomide intraperitoneally on the 8th day of gestation.

**Fig. E.** Alizarin-stained fetuses, 20 days. The one on the right shows absence of two thoracic vertebrae, all lumbar and sacral vertebrae, and two pairs of ribs. Its mother received 50 mg. of thalidomide intraperitoneally from the 8th to the 12th day of gestation. The fetus on the left is normal and is from a control litter whose mother received 0.3 c.c. of CMC intraperitoneally on the days of gestation mentioned above.

**Fig. F.** Alizarin-stained fetuses, 20 days. The one on the left is normal and is from a control litter whose mother received 0.3 c.c. of CMC intraperitoneally from the 8th to the 12th day of gestation. The fetus on the right has abnormal vertebrae: some centra are absent; others are fused with adjacent centra. Its mother received 50 mg. of thalidomide intraperitoneally from the 8th to the 12th day of gestation.

**Fig. G.** Alizarin-stained sterna of 20-day-old fetuses. The specimen on the left is normal and is from a fetus of a control litter whose mother received 0.3 c.c. of CMC intraperitoneally from the 8th to the 12th day of gestation. The sternum on the right is from a fetus whose mother received 50 mg. of thalidomide by the same route and on the same days. Note absence and ‘scrambling’ of sternebrae.
and 68.6 per cent. showed no ossification of the 5th sternebra (Text-fig. 3). No other defects were observed.

Comparable controls (4AC) had 119 young; only 4.2 per cent. had abnormal centra and 5.9 per cent. had no 5th sternal centre.

In the group treated by stomach tube (4BT), there were ninety-nine fetuses. The vertebral centra were malformed in 23.2 per cent. of fetuses and 11.1 per cent. showed no ossification of the 5th sternebra.

The control group for the above experiment (4BC), contained 105 fetuses. Only 1 per cent. had abnormal vertebral centra; no other defects were observed.

DISCUSSION

Various doses, methods and days of administration of thalidomide have been tried in these experiments in an attempt to produce congenital defects in the rat, especially of the limbs. Our results indicate that thalidomide does not usually produce serious gross anomalies in the Holtzman rat. With the exception of one fetus (Plate 1, Fig. B) which had severely malformed hind-limbs, fetuses did not have serious defects of limbs. Even in the above-mentioned case, alizarin staining revealed that the limb bones were of normal length and shape.

We are not certain that the clubbed feet and the abnormal flexion of toes recorded in Table 3 are genuine thalidomide abnormalities. These apparent defects may have resulted from the staining and dehydration techniques or from the effects of CMC.

There seems to be a slight increase in the resorption rate in Holtzman rats following the administration of thalidomide. However, there does not appear to be a relationship between the rate of resorption and the dosage, methods, or days of administration similar to that observed by Christie (1962a, b), Bignami et al. (1962) and Giroud et al. (1962) in various other strains of rat.

To our knowledge there has been no previous report on the sedating effect of thalidomide on the rat. In our Experiment IV, 500 mg. of this drug appeared to sedate the rats; lower doses had no apparent effect.

Our experiments show that thalidomide has a teratogenic effect on rat development, especially on the ossification of the sternum and vertebral column. Although there was absence of the 5th sternebra in some control fetuses, the treated ones showed a much higher incidence of the anomaly. Our experiments suggest that an increase in the dosage of thalidomide causes an increase in the number of missing sternebrae, or a delay in the ossification of the sternum. These findings are similar to those reported by Klein Obbink & Dalderup (1963). It may be that the absence of the 5th centre in treated fetuses represents only delayed ossification and that if the young were allowed to mature, complete ossification of the sternum might occur. The detection of delayed ossification of the sternum in control fetuses suggests that CMC may also affect ossification. However, if it does, its effect is not as pronounced as that of thalidomide. Our finding of
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'scrambled' sternabrae in some treated fetuses agrees with the observations of McColl et al. (1963).

To our knowledge there have been no other reports of thalidomide-treated rat fetuses exhibiting such severe abnormalities of the vertebral column. The fact that some control fetuses showed minor defects of the centrum, 'dumb-bell-shape' and/or duplication of the centrum, also suggests that CMC may be a weak teratogenic agent. The possibility of a genetic factor being responsible for this anomaly cannot be ruled out however. Other workers have not observed pronounced spinal defects in the rat, but DiPaolo (1963) and DiPaolo et al. (1964) have reported that thalidomide administered to mice will cause severe spinal abnormalities. Our results in the Holtzman rat are somewhat similar to those described by DiPaolo and his colleagues in mice.

Various investigations on rats suggest that thalidomide is strain-specific in its action, that is, its teratogenic effects vary in different strains. The 10th to 12th day of embryonic development seems to be the critical period for inducing skeletal anomalies in the Holtzman rat. Additional work is necessary, however, to determine if the vertebral and sternearal effects detected in these experiments are reliable criteria for detecting teratogenicity of drugs.

Although the congenital defects observed in the rat are different from those found in humans, the results show that thalidomide is a teratogenic agent as was strongly suspected. It is likely that thalidomide acts by blocking some metabolic process essential to cellular differentiation. The nature of the metabolic block affecting differentiation, especially of mesenchyme, is unknown. Various hypotheses concerning the mode of teratogenic action of thalidomide have been suggested. One suggests that thalidomide acts by antagonizing the metabolism of vitamins, especially those of the B complex (Evered & Randall, 1963; Felisati, 1964). Another contends that thalidomide is a glutamine antagonist (Bassil et al., 1962). Further studies are needed, especially biochemical, to detect the mode of action of thalidomide teratogenicity.

The design of the present experiments seems adequate for testing a drug for its possible teratogenic effect on skeletal structures. However, to test a drug's effect on other tissues and organs, histological preparations would also have to be made. Our investigations point out the need for special preparations for the detection of all effects of drugs.

SUMMARY

1. Pregnant Holtzman albino rats were given 25-, 50- or 500-mg. doses of thalidomide intraperitoneally, or by stomach tube, during the time their embryos' limbs were developing.

2. Except in rare instances, thalidomide had no effect on limb development. In one case, limb malformations closely resembling thalidomide deformities in man and rabbits were observed.
3. The commonest effect of thalidomide on rat development was absent, delayed, or abnormal ossification of the 5th sternebra.

4. The most serious abnormalities observed were maldevelopment, fusion or absence of vertebrae.

5. Other skeletal defects observed occasionally were: ‘scrambled’ sternebrae, abnormal flexion of toes, ‘clubfoot’, absence of vertebrae, ribs and tail and supernumerary ribs.

6. In general the 50-mg. dose of thalidomide induced the most serious defects. The method of administration (by stomach tube or by intraperitoneal injection) seemed to make no difference. The critical period during which thalidomide seems to act on the skeletal system in this strain of rat is from the 10th to 12th days of gestation.

7. Some control fetuses had minor skeletal defects suggesting that sodium carboxymethylcellulose may also be a weak teratogen in this strain of rat.

8. The thalidomide-produced vertebral and sternebral defects were not visible during macroscopic examination. Alizarin preparations were needed to demonstrate the defects; this suggests that the above technique should be a part of all investigations aimed at detecting teratogenicity of drugs.

RÉSUMÉ
Malformations squelettiques chez l'embryon de rat Holtzman, à la suite de l'administration de thalidomide

1. On a administré à ratteres albinos Holtzman gestantes, par voie intrapéritonéale, ou par tubage stomacal, des doses de 25, 50 ou 500 mg. de thalidomide, pendant la période de développement des membres des embryons.

2. Sauf en de rares cas, la thalidomide n'a pas d'action sur le développement du membre. Dans un seul cas, on a observé des malformations des membres ressemblant étroitement à celles que la thalidomide provoque chez l'Homme et le Lapin.

3. L'effet le plus fréquent de la thalidomide sur le développement du rat consiste en un retard, une absence ou des anomalies de l'ossification de la 5ème sternèbre.

4. Les anomalies les plus sérieuses observées sont un développement anormal, la fusion ou l'absence des vertèbres.


6. En général, la dose de 50 mg de thalidomide a induit les anomalies les plus graves. La méthode d'administration (par tubage stomacal ou par injection intrapéritonéale) n'a pas semblé apporter de différences. La période critique au cours de laquelle la thalidomide semble agir sur le système squelettique de cette lignée s'étend du 10e au 12e jour de la gestation.

7. Quelques foetus-témoins présentaient des anomalies mineures, suggérant
Skeletal malformations and thalidomide que la carboxyméthylcellulose sodique peut être aussi un agent tératogène faible pour cette lignée de rats.

8. Les anomalies vertébrales et sternébrales produites par la thalidomide n’étaient pas visibles macroscopiquement. Des préparations à l’alizarine étaient nécessaires pour les mettre en évidence; ceci permet de penser que cette technique devrait être intégrée à toutes les recherches destinées à déceler le pouvoir tératogène des substances chimiques.

REFERENCES


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