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The Teratogenicity of Cyclophosphamide in Mice[1,2]

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SUMMARY

Cyclophosphamide, administered intraperitoneally to time-dated pregnant Swiss Webster mice on gestational Days 9 through 14 in a dosage of 20 mg/kg, resulted in increased numbers of resorptions and a variety of teratogenic effects. Gross examination of fetuses revealed cleft palate, exencephaly, digital defects, and kinky tail. Skeletal anomalies included polydactyly, syndactyly, ectrodactyly, adactyly, fusion of the long bones, curvature of the long bones, and missing ribs. Soft tissue malformations included open eyes, aphakia, microphakia, hydronephrosis, and hydrocephalus. Dosages of 5 and 10 mg/kg resulted in increased resorption and decreased growth rates in a dose-related manner but produced no discernible anomalies. Fetal mortality curves were biphasic, with the highest peak occurring on administration Days 9 and 10 and a second peak on Days 13 and 14.

INTRODUCTION

Cyclophosphamide (Cytoxan®, Endoxan®) is a radiomimetic alkylating agent used in the treatment of neoplastic disease. Unlike the structurally related nitrogen mustards, cyclophosphamide is relatively inert *in vitro* (1). Studies on the biologic alteration of cyclophosphamide have resulted in the suggestion that nor-nitrogen mustard, β,β'-dichlorodiethylamine, is the active alkylating agent (12). The teratogenicity of nitrogen mustards has been demonstrated in amphibians (2, 6) and in rats (8, 11). Cyclophosphamide has also been reported to be teratogenic in rats. Single intraperitoneal injections of cyclophosphamide, 20 to 40 mg/kg, on gestational Days 10 through 15 were found to induce exencephaly, microcephaly, syndactyly, hexadactyly, and missing ribs in the offspring. The critical period was found to be Days 12 and 13. Resorptions due to the administration of cyclophosphamide were observed on Days 11 through 14 (14). In the rabbit the subcutaneous administration of 50 mg of cyclophosphamide per rabbit on Days 10 through 13 consecutively induced primarily cleft palate with associated hypognathus, resorptions, and decreased fetal size (3, 4). Injections of 300 mg/rabbit were lethal to the dam after the second injection; 100 mg/rabbit induced 100% fetal resorptions (3). The treatment of developing chicken eggs with cyclophosphamide has been found to induce deformities. The anomalies induced were dependent upon the day of incubation on which cyclophosphamide was administered and included primarily micromelia (5).

The purpose of the present investigation is to report the teratogenic action of cyclophosphamide in another mammalian species, the mouse. In addition to characterization of external and skeletal defects, the results of soft tissue examination are presented.

MATERIALS AND METHODS

Virgin Swiss Webster mice (obtained from Arthur Sutter Farms, Springfield, Mo.) were housed in groups of 10 in stainless steel "shoe-box" cages and allowed food and water *ad libitum*. Mating of females was accomplished by pen breeding, where males of demonstrated fertility and of the same strain were placed with females using one male for every five females. This procedure was carried out for five consecutive 16-hour periods (4 P.M.–8 A.M.) each week. Copulation was ascertained by the presence of the vaginal plug or by the finding of sperm in the vaginal smear when vaginal plugs were less conspicuous than usual. When plugs were found or when spermatozoa were present, the females were separated and housed in groups for treatment. This time was called Day 0 of pregnancy. Pregnant females were injected intraperitoneally with saline solutions of cyclophosphamide at dosages of 5, 10, or 20 mg/kg on one specific day of gestation (9 through 14); control pregnant mice were injected on a specific day of gestation with an equal volume of saline, 0.01 ml/gm. Under ether anesthesia, caeserian section was performed on Day 18 and the uterine horns were externalized and extended. The number and position of live, dead, and resorbed fetuses were noted. Fetuses were then removed, sacrificed by excess ether, dried on absorbent paper, and weighed on a Roller-Smith balance. Crown-rump and transumbilical distances (width of fetus at the level of the umbilicus) were measured with a vernier caliper.

Individual fetuses were examined carefully for external anomalies. Each litter was divided into two subgroups. One subgroup was fixed in Bouin's solution for two weeks. Fetuses from this subgroup were sectioned by hand into 2- to 3-mm sections and examined under a dissecting microscope for soft tissue anomalies by the method of Wilson (15). The other subgroup was fixed in 95% ethanol, cleared, and stained with alizarin red by the method of Hurley (9). Stained skeletons were examined and measured under a dissecting microscope fitted with a reticle. Long bone measurements were recorded in reticle units (10 reticle units equal 1.4 mm).

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Statistical analyses of body weight, body measurements, and bone length were made by the grouped t test. Statistical evaluation of frequencies of anomalies and resorption were made by the binomial expansion method (7). The level of significance was chosen as $P \leqq 0.05$.

RESULTS

Embryo and Fetus Death. Single intraperitoneal injections of cyclophosphamide to groups of pregnant mice on gestational Days 9 through 14 resulted in statistically significant increases in *in utero* deaths at dosage levels of 5, 10, and 20 mg/kg, as shown in Table 1. Treatment on Day 9 with 20 mg/kg of cyclophosphamide resulted in a mortality rate of nearly 100 percent whereas sham treatment on the same day resulted in only 2 percent *in utero* deaths. The clear dose-effect relationship is best seen on Day 10 where all doses were used. Mice treated on Day 12 showed the lowest degree of *in utero* mortality resulting in a biphasic mortality curve. The variation in resorptions observed in control groups treated with saline at different days of gestation were not significantly different from each other.

Body Weights and Measurements. Fetal body weights were reduced significantly from corresponding controls by cyclophosphamide treatment in a dose-related manner as shown in Table 2. The greatest effect was seen when the drug was given early during the gestational period. Crown-rump distance, also shown in Table 2, was particularly reduced on Days 12 and 13 even at the 10 mg/kg dosage. Transumbilical measurements (Table 2) were reduced by cyclophosphamide treatment but were generally more variable than body weight or length. These data indicate a decreased fetal growth in response to increasing dosages of cyclophosphamide.

Externally Visible Anomalies. A variety of anomalies in fetuses derived from mothers treated with 20 mg/kg of cyclophosphamide were readily detected by observation of the delivered fetuses. The frequency distribution is shown in Table 3. Open eyes was a frequent anomaly. A statistically significant incidence of this defect occurred when the drug was administered on Days 9 and 10, about six days before the formation of the eyelid (16). Open eyes were not observed in animals treated after Day 10. Anomalies of the digits show a day-dependent character in that polydactyly occurred with the greatest frequency from treatment on Day 9, syndactyly on Day 10, ectrodactyly (fewer than normal number of digits) on Day 11, and adactyly (absence of digits) on Days 11 and 12. Each of these peak occurrences is statistically significant. Other anomalies, such as hindlimb dorsiflexion, exencephaly, and kinky tails, also were noted at statistically significant levels on certain days of gestation. Cleft palate was apparent on gross examination, but was seen better on sectioning of the fetuses for soft tissue anomalies and was associated with shortening of the snout. Although statistically significant levels of cleft palate resulted from treatment of mothers on Days 9 through 12 of the test period, treatment on the earlier days produced a higher incidence of the defect (Table 3). These anomalies were not seen in fetuses delivered from mothers treated with 5 or 10 mg/kg of cyclophosphamide except for two cases of exencephaly. The only anomaly observed in control fetuses was also a case of exencephaly.

Soft Tissue Anomalies. Table 3 also shows the frequencies of soft tissue anomalies. Only the 20 mg/kg dosage of cyclophosphamide effected such changes. Aphakia or microphakia occurred less frequently than cleft palate but are more severe malformations. Statistically significant levels of aphakia were associated with injections of the test drug early in the gestational period (Days 9–11) whereas microphakia was associated with later treatment (Day 12). Hydrocephalus was observed in both gross examination and soft tissue examination. As shown in Table 3, hydrocephalus was separated into two types: (*a*) external hydrocephalus where there is an excessive amount of fluid between the brain and dura mater, and (*b*) internal hydrocephalus where there is an abnormal accumulation of fluid in the ventricular system of the brain. External hydro-

Table 1

Dose (mg/kg) i.p.	Day of administration	Number of pregnant females	Number of implantations	Number of resorptions	% resorptions (mean ± S.E.)	Significance[a]
0	9–10	5	49	1	2.0 ± 2.0	
	11–13	14	136	13	9.6 ± 2.5	
	14	8	84	1	1.2 ± 1.2	
5	10	5	54	11	20.4 ± 5.5	yes
10	10	6	49	18	36.8 ± 6.9	yes
	11	4	44	0	0	no
	12	8	74	4	5.4 ± 2.6	no
	13	9	78	16	20.5 ± 4.6	yes
20	9	10	89	86	96.8 ± 1.9	yes
	10	8	76	51	67.3 ± 5.3	yes
	11	9	106	50	47.2 ± 4.9	yes
	12	7	79	9	11.4 ± 3.6	no
	13	6	61	25	41.0 ± 6.3	yes
	14	9	94	16	17.0 ± 3.9	yes

The incidence of cyclophosphamide-induced fetal resorptions.

[a] Significantly different from the control group treated on the same day of gestation ($P \leqq 0.05$).

Table 2

Day of administration[a]	Measurement	Dose (mg/kg) i.p. 0	5	10	20
9	N	49			3
	B.W.	1.65 ± 0.05			1.13 ± 0.10[b]
	C.R.	31.7 ± 0.5	N.D.	N.D.	16.6 ± 7.1[b]
	T.U.	11.0 ± 0.2			10.0 ± 0.8
10	N	49	42	31	25
	B.W.	1.65 ± 0.05	1.51 ± 0.04[b]	1.17 ± 0.03[b]	0.90 ± 0.04[b]
	C.R.	31.7 ± 0.5	31.4 ± 0.3	28.5 ± 0.3[b]	22.8 ± 0.6[b]
	T.U.	11.0 ± 0.2	10.0 ± 0.2[b]	9.8 ± 0.2[b]	10.0 ± 0.4[b]
11	N	131		44	57
	B.W.	1.37 ± 0.02		1.03 ± 0.03[b]	0.77 ± 0.03[b]
	C.R.	30.4 ± 0.2	N.D.	25.9 ± 0.4[b]	21.2 ± 0.5[b]
	T.U.	9.9 ± 0.1		9.6 ± 0.3	9.2 ± 0.1[b]
12	N	131		70	70
	B.W.	1.37 ± 0.02		1.31 ± 0.03	1.07 ± 0.03
	C.R.	30.4 ± 0.2	N.D.	28.9 ± 0.2[b]	26.3 ± 0.5[b]
	T.U.	9.9 ± 0.1		10.5 ± 0.1[b]	9.9 ± 0.2
13	N	131		67	36
	B.W.	1.37 ± 0.02		1.32 ± 0.03	0.92 ± 0.02[b]
	C.R.	30.4 ± 0.2	N.D.	28.3 ± 0.4[b]	25.3 ± 0.4[b]
	T.U.	9.9 ± 0.1		9.6 ± 0.2	8.8 ± 0.1[b]
14	N	59			78
	B.W.	1.54 ± 0.02			1.34 ± 0.03[b]
	C.R.	28.5 ± 0.2	N.D.	N.D.	27.8 ± 0.2[b]
	T.U.	8.8 ± 0.1			8.5 ± 0.1[b]

The effect of cyclophosphamide on mouse fetal size. Values are mean ± S.E. in appropriate units: weight in grams, distance in millimeters. N, number of fetuses in the group; B.W., body weight; C.R., crown-rump distance; N.D., not determined; T.U., transumbilical distance.

[a] Day of gestation cyclophosphamide was administered.

[b] Significant difference from corresponding control at $P \leqq 0.05$.

cephalus occurred at statistically significant levels in fetuses from mothers treated on Days 11 or 12 of gestation, but internal hydrocephalus did not occur at statistically significant levels. Hydronephrosis was another severe malformation seen infrequently. Fetuses delivered from mothers treated with 5 or 10 mg/kg of cyclophosphamide showed two cases of microphakia out of 98 fetuses examined. No defects were observed during soft tissue examination of control fetuses.

Skeletal Defects. Cyclophosphamide (20 mg/kg) treatment of pregnant mice on Days 10 and 11 of gestation induced a wide variety of skeletal abnormalities in offspring (Table 3). Three types of anomalies were noted: absence or nonossification of ribs, fusion of the long bones, and digital defects. Fusion of long bones occurred in the forelimbs and the hindlimbs. In some cases, however, only the tibia and fibula were fused but this defect was not statistically significant. Curvature of the tibia or fibula was also seen. Curvature of bones was correlated with treatment on Day 12 of gestation and its frequency of occurrence is statistically significant. None of the above-mentioned skeletal anomalies were seen in alizarin red-stained control fetuses or from mothers treated with 5 or 10 mg/kg of cyclophosphamide.

The fetal long bones were shortened by cyclophosphamide in a dose- and day-dependent fashion as summarized in Chart 1. Even lower dosages of cyclophosphamide, i.e., 5 and 10 mg/kg, given on Day 10 effected statistically significant shortening of the long bones which was not detected by gross observation. Although maximal effects of drug treatment were produced when the agent was given early in gestation, treatment as late as Day 14 produced statistically significant shortening.

DISCUSSION

These experiments have shown that the administration of single intraperitoneal injections of cyclophosphamide into the pregnant mouse from the 9th through the 14th day of gestation induced fetal anomalies at dosages which yielded no toxic manifestations in the adult.[4] Anomalies of the soft tissues and skeleton as well as gross morphologic features are related to both the dosage and the day of administration of cyclophosphamide.

The induction of cleft palate by cyclophosphamide treatment on Days 9 through 12 but not on Days 13 or 14 is an inter-

[4] A dosage of 400 mg/kg of cyclophosphamide, administered intraperitoneally in saline solution, in a volume of 0.01 ml per gram of body weight, was not lethal to any of a group of 20 adult Swiss Webster female mice during a 72-hour observation period.

Table 3

Anomaly	Day of gestation on which cyclophosphamide was administered[a]					
	9	10	11	12	13	14
Open eyes	2/3[b]	19/25[b]	0/57	0/70	0/36	1/78
Polydactyly	2/3[b]	6/25[b]	3/57[b]	2/70[b]	0/36	0/78
Ectrodactyly	0/3	5/25[b]	32/57[b]	5/70[b]	0/36	0/78
Kinky Tail	0/3	0/25	25/57[b]	9/70[b]	0/36	0/78
Syndactyly	0/3	11/25[b]	0/57	0/70	0/36	0/78
Adactyly	0/3	0/25	4/57[b]	4/70[b]	0/36	0/78
External hydrocephalus	0/3	0/25	5/57[b]	2/70[b]	0/36	0/78
Internal hydrocephalus	0/1	4/13	2/29	1/34	0/17	0/35
Exencephaly	0/3	10/25[b]	8/57[b]	2/70[b]	0/36	0/78
Limb dorsiflexion	0/3	6/25[b]	0/57	4/70[b]	0/36	0/78
Cleft palate	1/1	8/13[b]	19/29[b]	13/34[b]	0/17	0/35
Aphakia	1/1	10/13[b]	6/29[b]	0/34	0/17	0/35
Microphakia	0/1	0/13	2/29	3/34[b]	0/17	1/35
Hydronephrosis	0/1	1/13	4/29[b]	0/34	0/17	0/35
Ribs absent or not ossified	0/2	2/11[b]	16/27[b]	0/35	0/18	0/36
Bone fusion—hind limb	0/2	3/11[b]	7/27[b]	0/35	0/18	0/36
Bone fusion—front limb	0/2	3/11[b]	1/27	0/35	0/18	0/36
Tibia and fibula fusion	0/2	1/11	1/27	0/35	0/18	0/36
Curvature of tibia and fibula	0/2	0/11	0/27	3/35[b]	0/18	0/36

Incidence of cyclophosphamide-induced fetal anomalies. (Single i.p. injections into pregnant mice, 20 mg/kg.) Values in the body of the table represent the number of animals with the particular anomaly per total number of fetuses in the group. These anomalies did not occur in fetuses from control groups or in fetuses from females treated with 5 or 10 mg/kg of cyclophosphamide with these exceptions: controls, one case of exencephaly (1/258); 10 mg/kg group, two cases of exencephaly (2/212) and two cases of microphakia (2/98).

[a] Pregnant females were sacrificed on the 18th gestational day.

[b] Significant difference from corresponding controls at $P \leqq 0.05$.

esting finding. The palate closes in the mouse at about Day 14 or 15; cyclophosphamide produces its effect at a time remote from closure but does not affect closure when given at the time of the event. Thus the action of cyclophosphamide is probably different from cortisone, which induces cleft palate near the time of palatine closure (10). In the rabbit, cyclophosphamide induces cleft palate when given consecutively on Days 10 through 13 of gestation (4). Therefore, it would appear that the rabbit is like the mouse in that cyclophosphamide administration in advance of palatine closure will affect normal development of the palate. A possible explanation is a slow metabolic change of cyclophosphamide, presumably by the mother, to a derivative which affects palate closure. The induction of open eyes by cyclophosphamide in the mouse followed a similar pattern. Open eyes may result from failure of eyelid formation, ablepharia (Day 16), or failure of the formed lid to close on Day 17–18. If the defect is failure of eyelid formation, cyclophosphamide or metabolic derivatives of cyclophosphamide must be present for several days before eyelid formation as the defect cannot be induced when cyclophosphamide is given as late as the eleventh day of gestation. Other soft tissue structures were not widely affected by cyclophosphamide. Those soft tissue structures which were affected, however, were severely malformed. Important defects were hydrocephalus and hydronephrosis.

The effects of cyclophosphamide on the development of the skeleton of the mouse are severe and include fusion and/or curvature of the long bones. The skeleton was particularly sensitive to cyclophosphamide at very low dosages. For example, 5 mg/kg effected (statistically significant) shortening of the long bones without other evident effects. Shortening of the long bones seems to be a very sensitive indication of growth inhibition. The drug effects on digital formation in mice show a high degree of day-dependency and follow an orderly progression with time: polydactyly, syndactyly, ectrodactyly and finally, adactyly.

Of interest to the present findings are the results obtained in cyclophosphamide teratology experiments conducted in another strain of mice: strain DM/MK. Late in the course of the present investigations a paper published in the Japanese literature (13) came to the authors' attention which demonstrates the teratogenic response of the DM/MK mouse to cyclophosphamide. The anomalies reported to be induced by cyclophosphamide in the DM/MK strain were cleft palate, syndactyly, and a statistically insignificant level of kinky tail occurrence. The dosages required to induce these anomalies in the DM/MK strain were considerably higher than dosages required in the Swiss Webster strain. In the DM/MK strain 25 mg/kg of cyclophosphamide was an ineffective teratogen, whereas 50 and 100 mg/kg subcutaneously produced the teratogenic effects when given daily during the period of the 11th to 14th days of gestation. Thus, another strain of mouse appears to be more resistant to the teratogenic effects of cyclophosphamide than the Swiss Webster mouse.

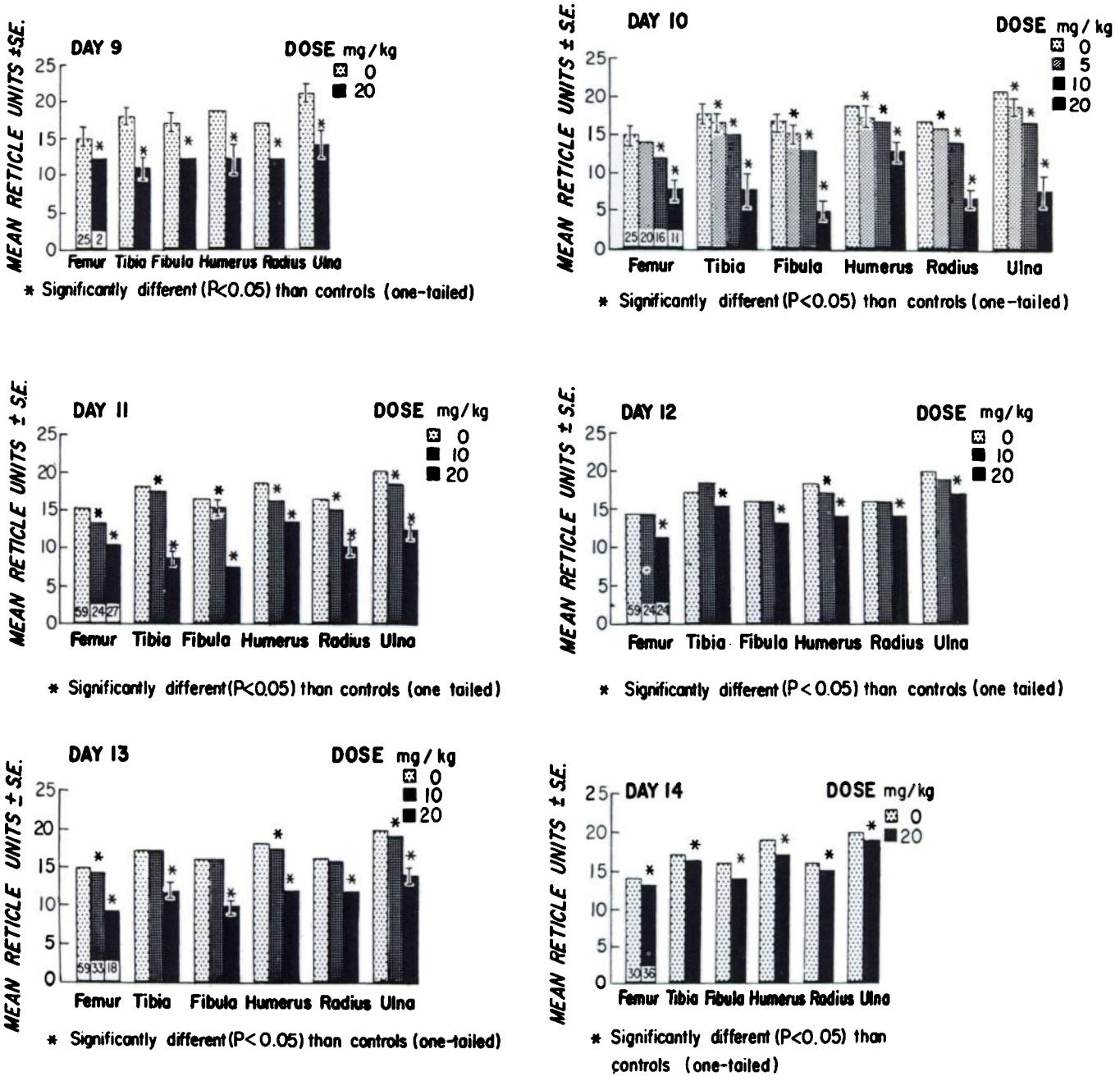

Chart 1. The effect of cyclophosphamide on the length of the long bones. The mean length ± S.E. is given on the ordinate in reticle units for each day cyclophosphamide was administered (10 reticle units equal 1.4 mm). The bones measured are shown on the abscissa and represent the measurement for the fetuses' right side. There was no significant difference between measurements of corresponding bones on the right or left side. The various dosages administered are shown by *stippling* of the vertical bars.

Cyclophosphamide demonstrates species differences as well as strain differences. Several differences between the teratogenic effects of cyclophosphamide in the present mouse studies and in rats (14) suggest that cyclophosphamide may be handled differently by mice and rats and/or the conceptuses of these two species. The mice data show an apparent biphasic distribution with respect to *in utero* mortality but in rats there is a single peak at Day 12 (14). Open eyes are found in mice and rabbits (3) but not in rats. Cleft palate induction by cyclophosphamide is not reported in rats (14). In addition, the effects of cyclophosphamide on the development of the skeleton are more severe in mice than in rats (14), where fusion or curvature of long bones was not reported.

The finding of cyclophosphamide teratogenicity agrees well

with the hypothesis that cyclophosphamide is biotransformed to nitrogen mustard (methchlorethamine) or nor-nitrogen mustard (nor-methchlorethamine), as put forth by the innovator of the compound (1) and others (12). The fact that the teratogenicity of cyclophosphamide in the rat (14) resembles that of nitrogen mustard (8) may be taken as indirect evidence that cyclophosphamide is biotransformed in the rat to nitrogen mustard or a derivative of nitrogen mustard. On the other hand, the teratogenic effects of cyclophosphamide may only coincidentally resemble those of the nitrogen mustards or be a property of the common bis(β-chlorethyl)-amino moiety of these molecules. The active components and/or metabolic transformation products of cyclophosphamide which produce teratogenic responses remain to be resolved.

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