Induction of Craniofacial Malformations in Rhesus Monkeys (Macaca mulatta) with Cyclophosphamide

HAROLD M. MCCLURE, D.V.M.
ANN L. WILK, Ph.D.
ELIZABETH A. HORIGAN, B.S.
ROBERT M. PRATT, Ph.D.
Atlanta, Georgia 30322

Administration of *cyclophosphamide* to pregnant *rhesus monkeys* induces two syndromes of *craniofacial malformations* characterized by underdevelopment of the face of different pattern depending on the timing of treatment. The first is *cleft lip* with *cleft palate* and exophthalmos. The second is a *craniofacial dysmorphia* with marked underdevelopment of the midfacial bones, highly arched closed palate, and either meningoencephalocele or persistent anterior fontanel. Since cleft lip and palate are among the most common malformations in man, and several human syndromes involving abnormal craniofacial development have features in common with the dysmorphic monkeys, these induced anomalies may prove valuable in investigations on the pathogenesis of such malformations and as surgical models.

Cyclophosphamide is used in the treatment of certain neoplastic diseases, as an immunosuppressive drug in transplant recipients, and in treatment of some forms of chronic renal connective tissue diseases (Lee et al., 1973; Schein and Winokur, 1975). The antineoplastic and cytotoxic activity has been associated with metabolites which are capable of alkylating cellular macromolecules rather than with the cyclophosphamide itself (Brock and Hohorst, 1963; Connors et al., 1974). Administration of this drug during organogenesis causes craniofacial, skeletal, and ocular malformations in chick embryos (Singh and Gupta, 1972), mice (Gibson and Becker, 1968; Metah et al., 1976), rats (Chaube and Murphy, 1968; Singh, 1971) and rabbits (Gerlinger, 1964). In some cases the malformations are very specific; for example, administration of cyclophosphamide and isoniazid to pregnant rabbits during the early part of organogenesis results in a high incidence of cleft lip and palate uncomplicated by other malformations (Wilk, unpublished data). Cyclophosphamide and drugs with alkylating activity are also suspected of being teratogens in man (Greenberg and Tanaka, 1964; Shotton and Monie, 1963; Diamond et al., 1960). In addition, also see Nicholson, 1968; Deuschle and Wiggans, 1953; Smith et al., 1958; Lee et al., 1962.

The purpose of this study was to evaluate the embryotoxic effects of cyclophosphamide in the rhesus monkey, an animal which, in general, resembles man in modes of maternal drug metabolism (Smith, 1969; Coulston and Serrone, 1969), placentation (Ramsey and Harris, 1966), and temporal scales of embryogenesis (Heuser and Streeter, 1941; Streeter, 1942, 1945, 1948, 1951; Heuser and Corner, 1957). However, we are not aware of any direct comparison of the metabolism of cyclophosphamide or its metabolites in rhesus monkeys and man. In addition, we sought to experimentally induce specific craniofacial malformations such as cleft lip and palate in monkey embryos in order to study the pathogenesis of malformations and to establish a nonhuman primate model for the plastic surgeon.

Dr. McClure is affiliated with the Pathology Department, Yerkes Primate Research Center, Emory University, Atlanta, Georgia 30322 and Dr. Wilk, Dr. Pratt and Ms. Horigan are affiliated with the Laboratory of Developmental Biology and Anomalies, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20014.

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Materials and Methods

Animals used in this study were feral rhesus monkeys that had been acclimated to laboratory conditions for approximately eight months prior to initiation of the study. The animals were maintained in single cage units and fed a standard laboratory diet supplemented with fruits and vegetables. Males were maintained in larger units that were utilized as breeding cages. Females were placed adjacent to the male on day 10 of the menstrual cycle (as determined by vaginal swabs) and were placed with the male for an eight-hour period on day 11 of the cycle. At the end of this eight-hour period, females were removed and returned to their home cage. Actual mating was usually confirmed by visual observation. The day of mating was listed as day 0 of gestation.

Females were checked for pregnancy on days 18, 21, and 24 by use of the "Sub-human Primate Tube Test for Pregnancy"[1] and on days 24 to 30 by rectal palpation. Pregnant animals were subsequently treated I.M. with cyclophosphamide[2] for various intervals between days 25 and 43 of gestation. Dosages varied from 2.5 mg/kg body weight to 20 mg/kg. Since isoniazid (INH), which is not teratogenic, enhances the teratogenic effects of cyclophosphamide in mice and rabbits (Wilk, unpublished data), it was often given in conjunction with cyclophosphamide. When used, INH (25 mg/kg) was given one hour before the cyclophosphamide. Twenty-four-hour urine samples were collected following each treatment, and serum was collected three hours after the last treatment for drug level determinations. A total of 53 timed pregnant females were used in this study.

During and following treatment, each animal was monitored closely for signs of toxicity or threatened abortion. Caesarean section was performed on day 60, 75, 150, or 158 of gestation. Each fetus was weighed, selected measurements were recorded, and they were examined for external developmental abnormalities.

[1] The Subhuman Primate Pregnancy Test Kits were provided by Dr. Gabriel Bialy, Contraception Development Branch, Center for Population Research, National Institute for Child Health and Human Development, National Institutes of Health, Bethesda, Maryland.

[2] Cytoxan, Mead Johnson Laboratories, Evansville, Indiana 47721.

Levels of cyclophosphamide and total alkylating metabolites were determined in serum of treated monkeys. The colorimetric method of Friedman and Boger (1961) as modified by Allen and Creaven (1972), involving alkylation of 4-(p-nitrobenzyl)-pyridine (NBP) to a colored quaternary pyridium state, was used to estimate alkylating metabolites which react spontaneously as well as cyclophosphamide which reacts with NBP after acid hydrolysis. Serum specimens were frozen immediately after collection and kept at −20°C until assayed. Ten milliliters of ice cold acetone was added to three mls. of serum, and the supernatant fraction was evaporated to dryness under a stream of filtered air and the residue dissolved in one ml. of distilled water. One half of the sample was analysed directly for alkylating metabolites. The remainder was hydrolysed after addition of 0.5 ml. of 1N HCl and boiling for 10 minutes. The sample was cooled, the pH adjusted to 4.6 with 1N NaOH and 0.2 M acetate buffer, pH 4.6, and then reacted with NBP for estimation of hydrolysed cyclophosphamide plus alkylating metabolites.

Results

Treatment of pregnant monkeys with 5–10 mg/kg of cyclophosphamide (with or without 25 mg/kg of isoniazid) resulted in two distinct types of craniofacial malformations (Table 1): cleft lip and/or cleft palate with ocular defects and craniofacial dysmorphia characterized by an underdevelopment of the midfacial bones and skull. Cleft lip/cleft palate was induced in four of 10 monkeys treated on days 27 and 29 of gestation. One animal treated at this time delivered a fetus with an abnormally elongated head and generalized subcutaneous edema; one fetus had a kinked tail; three animals aborted; and one delivered a normal fetus. Bilateral polysyndactyly of the feet was observed in one fetus with cleft lip and cleft palate. Cleft lip with or without cleft palate also occurred in two of four monkeys treated on days 27, 28, and 29 of gestation (Figures 1 and 2). One animal treated at this time aborted, and one delivered an infant with partially fused eyelids and multiple skeletal anomalies. The latter included fused ribs, absence of the left ulna, decreased numbers of carpal bones, and ectrosyndactyly of the left hand. The second syndrome, craniofacial dys-

TABLE 1. Gross Malformations Produced by Cyclophosphamide Treatment of Pregnant Monkeys.

drug treatment (mg/kg)		*gestation days treated*	*day of C-section*	*fetus weight (grams)*	*observations*
cyclo-P	*INH*				
10	25	27 + 29	75	31[1]	Cleft lip and palate-Open eyelids
10	25	27 + 29	74	25[1]	Cleft lip and palate-Open eyelids-Polysyndactyly
10	25	27 + 29	60	8	Narrow Head-S/Q Edema
10	—	27 + 29	74	24[1]	Cleft lip and palate-Open eyelids
5	—	27 + 28 + 29	75	35[1]	Cleft Lip
10	25	27 + 29	75	34[1]	Cleft Lip
7	—	27 + 28 + 29	158	360[3]	Cleft lip and palate[2]
7	—	27 + 28 + 29	158	380[3]	Skeletal Malformations-Eyelids Partially Fused
10	25	32 + 34 + 36	74	41	Craniofacial Dysmorphia-Meningoencephalocele-Ectrosyndactyly
7	25	32 + 34 + 36	150	330[3]	Craniofacial Dysmorphia[4]-Anterior Fontanel
10	25	34 + 35 + 36	74	33[1]	Craniofacial Dysmorphia
10	25	34 + 35 + 36	150	290[3]	Meningoencephalocele[5]-Cleft Palate-Ectrosyndactyly
10	25	36 + 38 + 40	74	22[1]	Craniofacial Dysmorphia
10	—	32 + 34 + 36	150	310[3]	Craniofacial Dysmorphia-Anterior Fontanel
10	—	32 + 34 + 36	150	308[3]	Meningoencephalocele
10	—	32 + 34 + 36	148	270[3]	Meningoencephalocele

[1] Growth retardation as compared to non-treated day 75 control fetuses which weigh 43.27 ± 3.49 (Kerr et al., 1969).

[2] Shown in figures 1 and 2.

[3] Growth retardation as compared to non-treated day 150 control fetuses which weigh 467.38 ± 42.76 (Kerr et al., 1969).

[4] Shown in figures 4 and 6.

[5] Shown in figure 5.

morphia (flattened nose bridge or "dish face") (Figures 3 and 4) and either meningoencephalocele (Figure 5) or persistent anterior fontanel (Figure 6) was induced in all eight fetuses delivered by animals treated with 7 or 10 mg/kg of cyclophosphamide at various times during days 32 and 40 of gestation. Ectrosyndactyly was present in two of these fetuses.

Treatment of pregnant monkeys with 20 mg/kg of cyclophosphamide caused *in utero* death or abortion in every case (Table 2). Chronic treatment for 10 days with 5.0 mg/kg of cyclophosphamide resulted in abortion or delivery of macerated fetuses. Normal fetuses were produced by three animals treated for six days with 2.5 mg/kg of cyclophosphamide. Single doses of 10–15 mg/kg did not induce malformations nor did any treatment after day 40 of gestation. Four of five animals treated twice on a single gestation day aborted; one of these animals had a normal fetus. Drug treatments that did not produce oral facial malformations are listed in Table 3.

The time course of cyclophosphamide and alkylating metabolites in serum shows a rapid depletion of drug three hours after administration, with only trace amounts of alkylating metabolites detectable at eight hours (Table 4). Pretreatment with isoniazid prolongs the level of cyclophosphamide in serum. Thus cyclophosphamide accounted for about 50% of the compounds measured three hours after administration of isoniazid and cyclophosphamide, whereas only about 15% was unchanged cyclophosphamide when the drug was given alone. The effect of isoniazid pretreatment on the outcome of pregnancy is unclear. Malformation and abortion were ob-

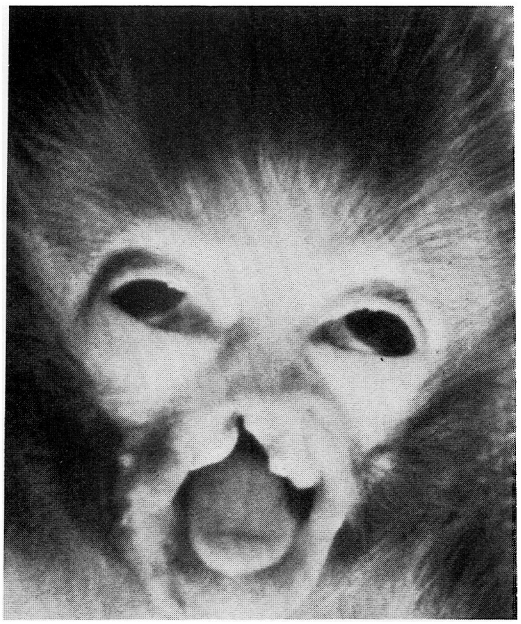

FIGURE 1. Close-up view of facial region of 5 week old rhesus infant showing cleft lip. The infant's mother was treated with cyclophosphamide (7 mg/kg) on day 27, 28, and 29 of gestation.

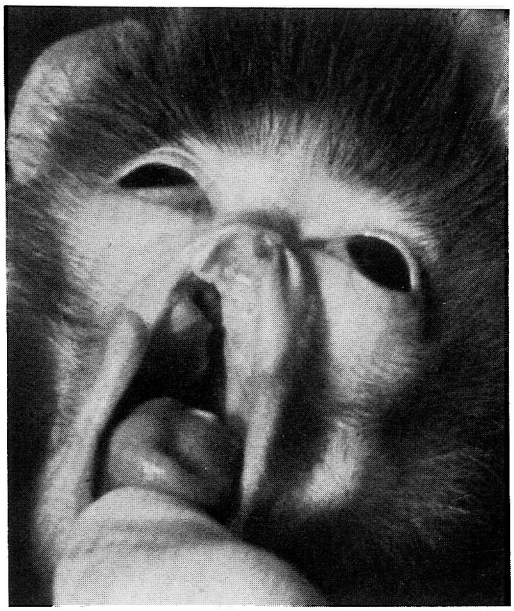

FIGURE 2. Close-up view of same infant showing cleft palate. Infant also has hypertelorism and divergent eye position.

served after treatment with cyclophosphamide, with or without INH (Tables 1 and 2). However, most of the monkeys in this study were pretreated with isoniazid and it is not known if the same incidence of malformations would have occurred with cyclophosphamide alone.

Analysis of serum from pregnant monkeys receiving similar dosages of cyclophosphamide (Table 5) did not reveal a relationship between the levels of cyclophosphamide or alkylating metabolites and malformations. Similarly, urinary excretion of compounds at 24-hour intervals over the course of treatment did not show a consistent pattern and could not be used to predict the outcome of pregnancy.

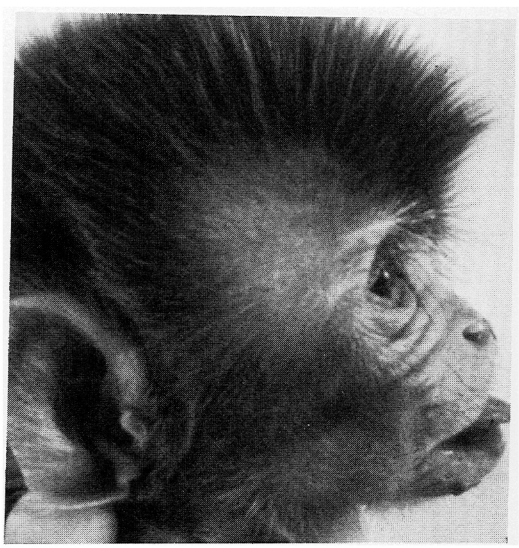

FIGURE 3. Facial features of a normal non-treated control infant for comparison with infant in figure 4.

Discussion

Results of this study clearly indicate that cyclophosphamide treatment is embryotoxic in rhesus monkeys. Although Wilson (1971) has noted that, in monkeys, embryotoxicity is more often manifested by early embryolethality and only rarely by teratogenicity, we have noted a high incidence of teratogenicity and a near normal incidence of *in utero* death or abortion in rhesus monkeys treated during the early stages of gestation with cyclophosphamide at a dosage of 10 mg/kg or less. Treatment with higher doses or for longer periods of time was 100% embryolethal. A high inci-

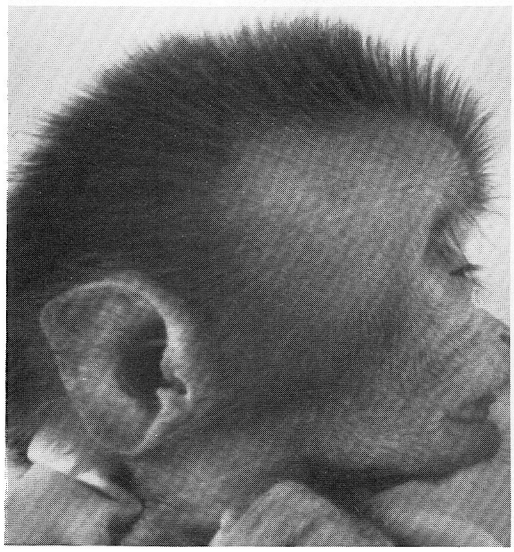

FIGURE 4. Facial features of infant with craniofacial dysmorphia. This infant's mother was treated with INH (25 mg/kg) and cyclophosphamide (7 mg/kg) on day 32, 34, and 36 of gestation. Note small head, frontal bossing, underdevelopment of the mid-face with concave appearance, ptosis of the eyelid (bilateral and symmetrical) and slight micrognathia.

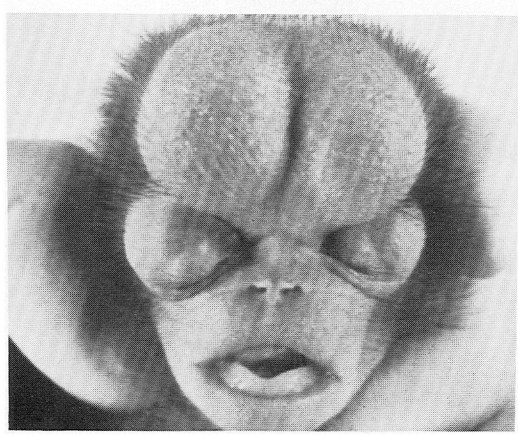

FIGURE 5. Facial features of infant with craniofacial dysmorphia, meningoencephalocele, cleft of the posterior part of the palate, and ectrosyndactyly. This infant's mother was treated with INH (25 mg/kg) and cyclophosphamide (10 mg/kg) on day 34, 35 and 36 of gestation.

dence of cleft lip, cleft palate and ocular defects was evident in animals treated on days 27 and 29 or days 27, 28, and 29 of gestation. Treatment between day 32 and day 36 of gestation resulted in a high incidence of craniofacial dysmorphia, faulty development of

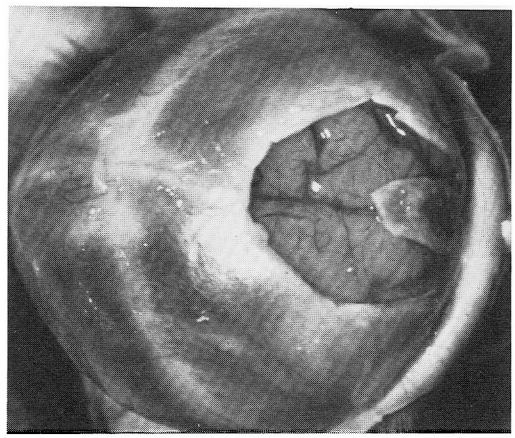

FIGURE 6. Photograph at autopsy of infant in figure 4 showing the lack of anterior skull formation; this area was covered by meninges and skin.

the anterior skull and meningoencephalocele. Polysyndactyly was evident in one animal in the former treatment group and two animals in the latter group showed ectrosyndactyly.

These anomalies presumably arise from deleterious effects at specific developmental stages and are analogous to gross malformations produced in rodents and rabbits (Gibson and Becker, 1968; Fritz and Hess, 1971; Manson and Smith, 1977). Treatment with cyclophosphamide near the beginning of organogenesis results in cleft lip and palate and polysyndactyly of the hind limbs, while treatment at the midpoint of organogenesis results in encephalocele, isolated cleft palate and ectrosyndactyly.

Macaques have been reported to respond similarly to man to a number of teratogens, such as thalidomide, X-irradiation, and androgenic hormones (Wilson and Gavan, 1967). Various alkylating agents, including nitrogen mustard, tretamine, chlorambucil, busulfan, and cyclophosphamide, have been suspected of causing either *in utero* death or developmental anomalies in humans (Nicholson, 1968; Shotton and Monie, 1963). Greenberg and Tanaka (1964) found multiple anomalies in a human infant whose mother was treated with cyclophosphamide throughout pregnancy. These anomalies included flattened nasal ridge, a groove on each side of the midline of the palate, ectrosyndactyly, hypoplastic middle phalanx of the fifth finger, and

TABLE 2. Cyclophosphamide Treatments Associated with Abortion or In Utero Death.

Drug treatment (mg/kg)		*gestation days treated*	*observations*
cyclo-P	*INH*		
20	25	30 + 32 + 34	Abortion
20	25	30 + 32 + 34	In Utero Death
20	25	30 + 32 + 34	Abortion
20	25	34 + 35	In Utero Death
5	25	26 thru 35	Abortion
5	—	27 thru 36	Abortion
10	25	28 + 30 + 32 + 34	Abortion
5	—	28 + 29 + 30	Abortion
7	25	28 + 30	Abortion
10	—	27 + 29	Abortion
10	25	26 + 28	Abortion
10	25	27 + 29	In Utero Death
10	—	27 + 29	Abortion
7	—	29 (2X[1])	Abortion
7	—	27 (2X[1])	Abortion
7	25	27 (2X[1])	In Utero Death
7	15	29 (2X[1])	Abortion
15	25	27	Abortion
5	25	27 + 28 + 29	Abortion
7	—	41 + 42 + 43	In Utero Death
7	—	28 + 29 + 30	Abortion

[1] Treated in morning and afternoon of the day indicated.

TABLE 3. Cyclophosphamide Treatments That Did Not Produce Oral Facial Malformations.

Drug treatment		*gestation days treated*	*day of C-section*	*fetus weight (grams)*	*observations*
cyclo-P	*INH*				
10	25	25 + 27	76	30[1]	Kinked Tail
7	25	28 + 29	77	43	Normal
5	25	28 + 29	75	36[1]	Normal
10	25	27	75	40	Normal
10	—	27	75	32[1]	Normal
10	25	27 + 29	75	36[1]	Normal
10	25	38 + 40 + 42	75	29[1]	Normal
10	25	39 + 41 + 43	75	33[1]	Normal
7	15	28 (2X[2])	75	32[1]	Normal
15	25	29	77	35[1]	Normal
15	25	28	77	35[1]	Normal
5	—	26 + 27 + 28	75	42	Normal
10	—	27 + 29	75	25[1]	Kinked Tail
2.5	—	25 thru 30	77	47	Normal
2.5	25	25 thru 30	75	38	Normal
2.5	—	25 thru 30	75	43	Normal

[1] Growth retardation as compared to non-treated controls.
[2] Treated in morning and afternoon of the day indicated.

TABLE 4. Time Course of Cyclophosphamide and Total Alkylating Metabolites in Serum After a Single IM Dose of 10 mg/kg Cyclophosphamide (With or Without 25 mg/kg Isoniazid) to Nonpregnant Monkeys.

treatment	*time*	*total compounds* μg/ml	*cyclophosphamide*		*alkylating metabolites*	
			μg/ml	*% of total*	μg/ml	*% of total*
Cyclophosphamide + Isoniazid	1 hr.	9.1	5.0	53	4.4	47
		8.0	3.8	48	4.2	52
		10.5	7.3	69	3.2	31
	3 hrs.	2.2	1.0	45	1.2	55
		2.4	1.1	46	1.3	54
		2.4	1.4	60	1.0	40
	8 hrs.	0.8	0	0	0.8	100
		0.6	0	0	0.6	100
		0.5	0	0	0.5	100
Cyclophosphamide	1 hr.	9.8	5.6	57	4.1	43
		8.0	4.4	54	3.7	46
		7.1	4.2	58	3.0	42
	3 hrs.	1.5	0.3	18	1.2	82
		1.3	0.1	11	1.2	89
		0.6	0.2	17	0.4	83
	8 hrs.	0.8	0	0	0.8	100
		0.8	0	0	0.8	100
		0.2	0	0	0.2	100

[1] Values derived from three monkeys.

TABLE 5. Pregnancy Outcome and Concentration of Cyclophosphamide and Alkylating Metabolites in Serum Three Hours After the Final Dose of Cyclophosphamide to Pregnant Monkeys.

animal number	*observation*	*drug treatment (mg/kg)*		*gestation days treated*	*total compounds* μg/ml	*serum levels*	
		INH	*cyclo-P*			*% cyclo-P*	*% metabolites*
32	Normal	25	7	28 + 29	3.5	37	63
40	Normal	25	10	27 + 29	8.0	39	61
26	Malformed	25	10	27 + 29	2.2	46	54
35	Malformed	25	10	27 + 29	4.3	60	40
34	Malformed	25	10	27 + 29	3.9	59	41
27	Malformed	—	10	27 + 29	2.1	21	79
41	Abortion	25	10	26 + 28	2.7	75	25

bilateral inguinal hernias. Toledo et al. (1971) recorded the complete absence of toes and a single coronary artery in an infant whose mother had been treated with cyclophosphamide and irradiation. Diamond and associates (1960) reported microphthalmia, cleft palate, and corneal opacity in an infant whose mother was treated with busulfan and irradiation. These craniofacial abnormalities and digital anomalies are comparable to the malformations we observed in monkey fetuses.

Cell death or tissue necrosis occurs in facial areas and limbs of rodents treated with cyclophosphamide (Eto, 1978; Schweichel and Merker, 1973) and may be causally related to malformations of these regions. Cyclophosphamide is not likely to be teratogenic itself, because it is not a cytotoxic or alkylating

agent (Hill et al., 1974; Connors et al., 1974). Recently Manson and Smith (1977) demonstrated that 4-ketocyclophosphamide, an alkylating derivative of cyclophosphamide, causes malformations in cultured mouse limb buds which were similar to limb malformations found after cyclophosphamide treatment *in vivo*. In addition to 4-ketocyclophosphamide, several other alkylating metabolites of cyclophosphamide have been identified, including, carboxyphosphamide, phosphoramide mustard and nornitrogen mustard (Hill et al., 1972; Colvin et al., 1973; Struck et al., 1975). Phosphoramide mustard and nornitrogen mustard possess potent cytotoxic/antitumor activity in a variety of cells in culture, whereas 4-ketocyclophosphamide and carboxyphosphamide (the major urinary metabolites) generally have low cytotoxic activity (Struck et al., 1971, 1975; Sladek, 1973; Connors et al., 1974; Friedman et al., 1976). We have evidence which suggests that phosphoramide mustard and nornitrogen mustard are major teratogenic compounds in rodents (unpublished data). In view of the complexity of cyclophosphamide metabolism and low cytotoxicity of major alkylating metabolites, it is not surprising that we failed to observe a correlation between embryotoxicity and maternal serum and urine levels of cyclophosphamide or alkylating derivatives.

The results of our studies and those of previously reported teratology studies in macaques indicate that this species is an appropriate experimental animal for evaluating drugs that are suspected of being teratogenic in man. The high frequency of cleft lip and cleft palate and craniofacial dysmorphia in animals treated with cyclophosphamide during specific times of gestation also indicates that this is a reproducible phenomenon, and suggests that it may provide animal models for reconstructive surgery. In this regard, it is noteworthy that syndromes of human malformation, for example, acrocephalosyndactyly (Apert syndrome), oral facial digital syndrome, and Larsen's Syndrome (Gorlin et al., 1976; Rimoin and Edgerton, 1967) present some features similar to anomalies produced in this study.

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Reprints: *Dr. Harold M. McClure*
Yerkes Primate Research Center
Emory University
Atlanta, Georgia 30322

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