Effect of Cyclophosphamide (Endoxan-Asta) on Palate Development in Albino Rats

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The science of the experimental induction of congenital malformations is increasing in importance, undoubtedly because of growing awareness that potential sources of non-hereditary danger to the developing embryo are many and frequently observed. Abnormal development of palate has been induced by a large variety of factors (Radiation, Hormones, Vitamins, antimetabolites, cytotoxic agents, salicylates, amniocentesis etc.) exerting their teratogenic influence during the critical phase of embryogenesis in various laboratory animals. Cyclophosphamide a potent cytostatic agent belonging to the alkylating group (Nitrogen mustard) has been recently synthesized by Arnold and Bourseaux (1958) where the active chemotherapeutic radical has been combined with a chemical group having selective affinity for rapidly growing normal and neoplastic cells in an attempt to overcome the pronounced haemodepressant effect of the alkylating agent. Since its introduction in 1958, it has been widely used in the treatment of various forms of cancer and most effectively in acute childhood leukaemia (references by Chaube et al, 1967). After demonstration of its teratogenic effects in chick embryos by Gerlinger et al (1963), cyclophosphamide has been subsequently shown to be teratogenic in various other laboratory animals (Kreybig, 1965; Chaube et al 1967, Gibson & Baker, 1968; Singh, 1971; and Singh et al, 1971). The various harmful influences (hereditary and nonhereditary produce such a great variation in the induced defects that the incidence of offspring with cleft palate in mice has ranged from a few per cent to almost one hundred percent (Kalter, 1959). The present study demonstrates variations in the abnormally developed palate in rats, induced by the cyclophosphamide.

Material and Methods

Sixtyone female rats (Wistar strain, weighing about 200 gm.) in oestrus were mated overnight with the fertile males of the same stock. The onset of gestation was determined by finding the spermatozoa in the vaginal smears and the following day was designated as the first day of gestation. On a day previously selected for treatment, the experimental rats were injected with a single intraperitoneal (I.P.) injection of the drug (freshly prepared in physiological saline) in various doses on the basis of mg/kg body weight from 12th through 16th day of gestation.

The control rats were given similar

quantities of physiological saline without the drug. The rats were sacrificed on 21st day of gestation (one day before delivery) and their uteri exposed and examined for live and dead foetuses and resorbed implantation sites. Live foetuses were removed, weighed and examined for external malformation (Singh, 1971 and Singh & Sanyal, 1972). Presence of deformed palate was confirmed under the dissecting microscope. Some of the foetuses showing defective palate formation along with controls were fixed in Bouin's solution, embeded in paraffin, sectioned and stained with haematoxylin and eosin.

Observations

As seen from table I, no deformity of the palate was detected in any of the foetuses from the control group or the one treated on 16th day of gestation. Whereas highly arched palate was noted in all the foetuses treated on the 15th gestation day, none showed the actual cleft. Varying degrees of cleft and deformed palates (Fig. 1 & 2) were observed in the groups treated from 12th to 14th day of gestation. Maximum incidence of cleft palate (70%) was observed in the 13th day group as compared with 27% and 42% found on 12th day and 14th day groups respectively.

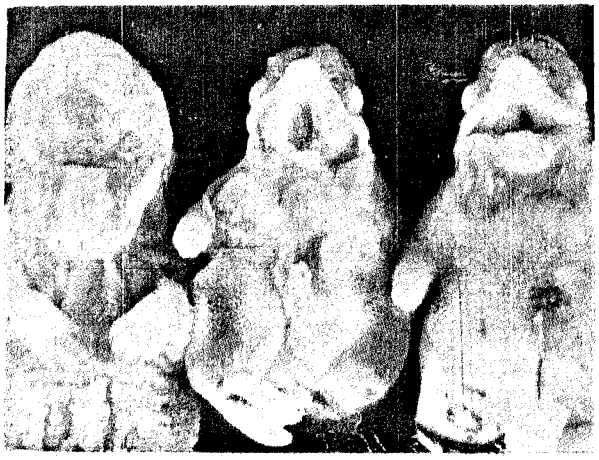

Fig. 1—Twentyfirst day foetuses from rats treated with Cyclophosphamide. A: Control. B; C. foetuses from rats given single I/P injection of Cyclophosphamide. B: (8 mg/12th day); shows slight degree of cleft palate, C: (15 mg/13th day) shows marked degree of cleft palate.

With increase in the dose of the drug on each day of treatment the incidence of the induced defect showed variation in the first three groups only (Table II). In these, 12th-14th days treatment groups, the higher dose in general produced higher incidence of the cleft palate. In the 15th day group, however, all doses employed produced highly arched palate in 100% of cases.

Histological examination did not show the presence of the cleft suspected in the cases of highly arched palates. However, the deformity was obvious (Fig. 4) and upward bulge of the tongue was noticeable in these cases. No fusion of tongue with palatine shelves or oral mucosa was observed in any case of cleft or deformed palates.

Discussion

Cleft palate is a common sequel to a variety of experimental teratogenic procedures though the incidence and severity varies. Incidence varies even with the same procedure used in the same species of animals. Some of the factors responsible for these variations can be divided into predisposing elements and precipitating elements. The former are more diverse and have both hereditary and environmental components i.e. genetic constitution of the mother and genetic constitution of the foetus (Fraser and Fainstat, 1951), time during pregnancy when the teratogen is

Table I

Incidence of Cleft Palate Induced by Cyclophosphamide given on different days of Gestation in Rats

Day of Treatt	No. Rats	No. Implants	No. Foetuses Examined	Normal Palate	Highly Arched Palate	Cleft Palate
12th	14	118	42	1 (2%)	30 (71%)	11 (27%)
13th	8	67	60	3 (6%)	13 (24%)	37 (70%)
14th	11	86	74	—	43 (58%)	31 (42%)
15th	10	60	60	—	60 (100%)	—
16th	8	66	66	66 (100%)	—	—
Controls	10	84	84	84 (100%)	—	—

Table II

Incidence of Cleft Palate Induced by Different doses of Cyclophosphamide on Various Days of Gestation in Rats

Day of Treatt.	Dose per Kg.	Foetuses Exam.	Normal Palate	H. Arched Palate	Cleft Palate
	8 mg.	28	1 (3%)	22 (79%)	5 (18%)
12th day	10 mg.	6	—	6 (100%)	—
	12-15 mg.	8	—	2 (25%)	6 (75%)
Total	8-15 mg.	42	1 (2%)	30 (71%)	11 (27%)
	10-12 mg.	24	3 (13%)	13 (54%)	8 (33%)
13th day	15 mg.	12	—	—	12 (100%)
	18 mg.	17	—	—	17 (100%)
Total	10-18 mg.	53	3 (5%)	13 (25%)	37 (70%)
	12 mg.	24	—	22 (92%)	2 (8%)
14th day	15 mg.	33	—	12 (36%)	21 (64%)
	18-20 mg.	17	—	9 (53%)	8 (47%)
Total	12-20 mg.	74	—	43 (58%)	31 (42%)
	15-16 mg.	4	—	4 (100%)	—
15th day	17-18 mg.	34	—	34 (100%)	—
	20 mg.	22	—	22 (100%)	—
Total	15-20 mg.	60	—	60 (100%)	—
	20 mg.	19	19 (100%)	—	—
16th day	25 mg.	39	39 (100%)	—	—
	30 mg.	8	8 (100%)	—	—
Total	20-30 mg.	66	66 (100%)	—	—

administered (Fraser et al, 1954), maternal weight and foetal weight (Kalter, 1956) and parity (Kalter and Fraser, 1953). The effectiveness of the precipitating element, when all other variables are kept constant, depends on the dose. Too small a dose has no discernible effect on the foetuses and a too large one destroys all embryos. The teratogenic dose is the one which induces anomalies, the frequencies of which vary with the dose within a limited range. Whereas Chaube et al (1967) found cleft palate in 3 out of 29 foetuses examined when cyclophosphamide was given on 11th day of gestation in rats, all embryos were found resorbed when this drug was given on 11th day of gestation by Singh (1971). On the other hand Chaube et al (1967) did not find any cleft palate by treatment on 12th day of gestation but the present study has shown it to be common in 12th-14th day treatment (27%-70%). Kreybig (1965) reported anomalies in rats with 5 mg of cyclophosphamide on 10th & 12th gestation day but the same dose resulted in all normal embryos in the 11th day treated group. As the variations in results of teratogenecity not only depend on the species but even on the strains of the animals (Cahen, 1964), such differences with cyclophosphamide are possibly due to different strains of the animals used.

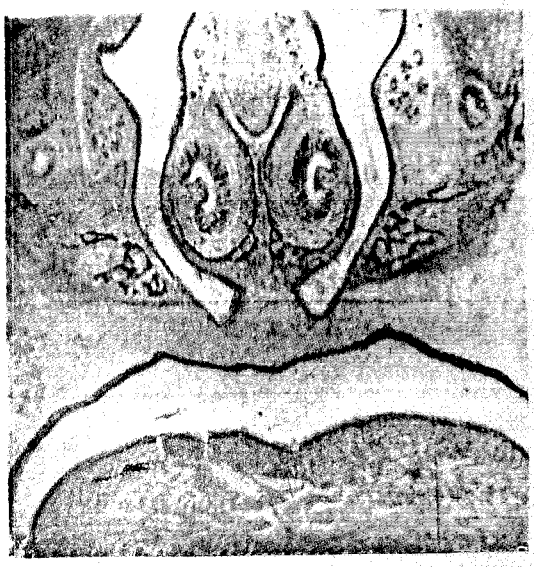

Fig. 3—Photomicrograph of twentyfirst day foetus from control rat, showing normally formed palate. Note the flatness of the tongue.

Though cleft palate is one of the commonest and important congenital anomalies, yet

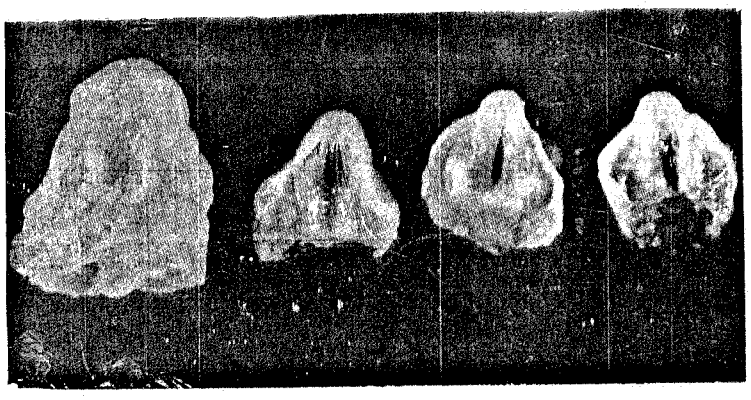

Fig. 2—Under surface of the palates from the heads of twentyfirst day foetuses from rats treated with cyclophosphamide. A: Control. B, C, & D. foetuses from rat given single I/P injection of cyclophosphamide, B: (15 mg/12th day) shows highly arched cleft palate; C: (8 mg/12th day) shows slight degree of cleft palate; D: (15 mg/13th day) shows complete cleft palate.

the evidence of the embryological mechanisms by which it occurs is scattered, scanty and sometimes conflicting. The palate closure occurs at a stage when most organogenetic processes are over. The palatine shelves, which eventually form the roof of the mouth, first appear as downgrowths from the inferior surface of the maxilla on either side of the tongue as if compressing it. In order to close, the shelves must change their position from the vertical to the horizontal plane, which requires displacement of the intervening tongue. Palatal shelves which develop their own force (Shelf force) to move them inwards against the resistance of the intervening tongue, move medially first in the posterior part and then proceed anteriorly. Prior to movement of the palatal shelves into the horizontal position the upper face is prognathous relative to the lower jaw but the maxillomandibular profile relation is reversed with the onset of horizontal shelf positioning. Tip of the tongue which lies behind the primary palate, slides forwards below it. Forward growth of the mandible promotes this forward and downward movement of the tongue. After both shelves become horizontal, they become more flattened and their free edges approach one another followed by epithelial fusion. The septum formed by the apposed epithelia breaks down and mesenchyme of the shelves becomes continuous.

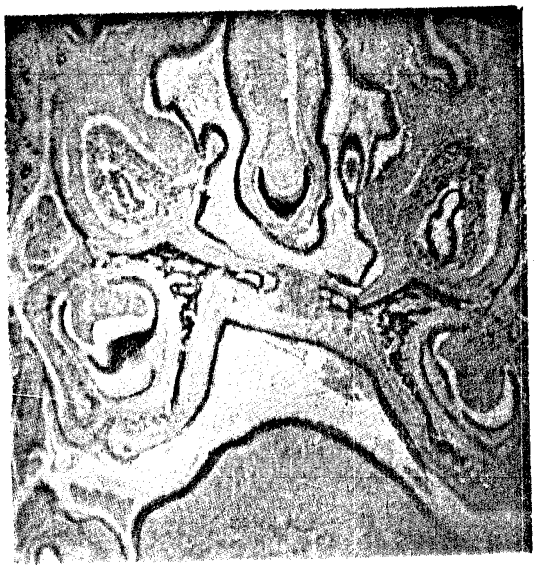

Fig. 4 Photomicrograph of twentyfirst day foetus from rat treated with (15 mg/12th day) cyclophosphamide showing highly arched palate. The upward buldge of the tongue is clearly seen.

There is a rapid synthesis of mucopolysaccharides on the apposing border of the palatine shelvss prior to their fusion (Larsson, 1960). Teratogens like cortisone (Fraser et al, 1954) and salicylates (Warkany & Takacs, 1959) which decrease this synthesis result in cleft palate. However, with glucocorticoids which also inhibit synthesis of mucopolysaccharides and induce cleft palate, no such correlation was found by Andrew and Zimmerman (1971). Antimitotic agents eg. X-rays (Hicks, 1953), nitrogen mustard (Haskin, 1948), 6 aminonicotinamide (Chamberlain & Nelson, 1963) possibly adversely affect the shelf force and cause cleft palate, or by their cytostatic action depress growth of the cranial base which may affect the shelf movement. Tongue may be crammed between the shelves in oligohydramnios, produced experimentally by amniocentesis (DeMyer & Baird, 1969). Micrognathia may come in the way of the forward movement of the tongue resulting in cleft palate. Theoretically an unduly large tongue could present greater tongue resistance and delay shelf movement but no example in experimental teratology is known so far. Displacement of the tongue has also been shown to be dependent on the

hyperextension of the head (Walker, 1969) which had been demonstrated on the screen as well (Walker, 1968). Cleft palate could result from interference wi h this mechanism e.g. in cervical vertebral deformities or in oligohydramnios or in foetal muscle degeneration. However, after induced paralysis of the muscles in the foetuses (Jacobs, 1971) no cleft palate resulted. The palatine shelves themselves may be hyperplastic and may have abnormal cartilage and bone formation interfering with closure e.g. in hypervitaminosis A (Kochhar & Johnson, 1965), or the shelves may be fused with the tongue e.g. in cases of meclizine hydrochloride treatment (Kendrick & King, 1964).

Palate closure therefore is a complicated process involving synchronised interactions of shelf, tongue, jaw and head. Major interference at various points of the system either by specific environmental agents or by mutant genes can bring about the failure of closure. Cyclophosphamide after its activation in the host tissue (Brock, 1967) has its major action related to the inhibition of deoxyribonucleic acid (DNA). The prolonged inhibtion of DNA synthesis may lead to localised cell death sufficient to upset the proliferative rates within the embryo (Ritter et al, 1971). The protruded thick tongue with shortened lower jaw are the important features of the syndrome of head anomalies produced by cyclophosphamide (Singh, 1971). The brachygnathia and macroglossia caused by cyclophosphamide may also account for the failure of closure of the palate in the present cases besides the arrested growth of the palatine shelves.

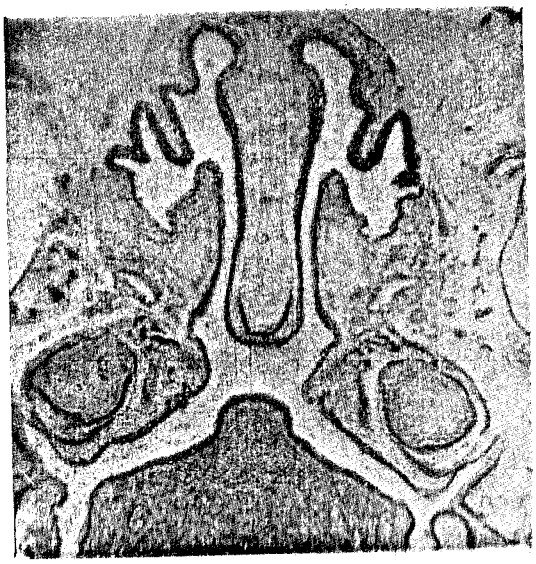

Fig. 5—Photomicrograph of twentyfirst day foetus from rat treated with (15 mg/13th day) cyclophosphamide showing complete cleft palate.

Though there are species differences in the morphology of the face in early development, yet mouse, rat and human embryos are remarkably similar in size when the face is forming and the process of palate formation appears basically to be the same in these mammalian species (Fraser, 1968). Efforts to reduce the frequency of cleft palate in man should be directed not only to a better understanding of the process of closure or to the further identification of specific environmental teratogens, but also to learning how to promote early shelf closure, and thereby prevent the possibility of this abnormality.

Summary

Single intraperitoneal injection of varying doses of Cyclophosphamide (Endoxan-Asta) was given to 51 pregnant albino rats of Wistar Strain during 12th through 16th days of gestation (the day of sperm positive vaginal smear designated as zero day of pregna-

ncy). Ten pregnant rats were used as controls where similar amount of saline was injected without the drug. Rats were sacrificed on 21st day of gestation and foetuses removed after uterotomy and examined under the dissecting microscope for presence of any cleft palate. Highly arched palate showing defective closure was observed in most of the litters where injections were given on 12th-15th days of gestation while rats injected on 16th day of gestation showed normal palate in all the litters. Varying degrees of actual cleft in the palate was found in 27% of foetuses treated on 12th day, 70% in those of 13th day treatment and 42% in case of 14th day group. No cleft palate was detected in the 15th & 16th day treated foetuses or in the control ones, though the 15th day treated group showed high arched palate in 100% of the foetuses. Mechanism of the production of the developmental defect has been discussed.

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