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DIGITAL ANOMALIES INDUCED BY CYCLOPHOSPHAMIDE (ENDOXAN-ASTA) IN RAT FOETUSES

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Malformations of the limbs including digits have been produced experimentally in mammalian foetuses by using a variety of treatments during gestation, e.g. *X-irradiation* (Murphy & de Renyi 1930, Warkany & Schraffenberger 1947, Russell 1950, Hicks 1953, Neifach 1960, Murakami et al. 1963, Nogami 1964); *nutritional deficiencies* (Warkany & Nelson 1941, Warkany & Schraffenberger 1943, Shepard et al. 1968); *hypervitaminosis A* (Cohlan 1953, Kalter & Warkany 1961, Barrow & Rowland 1969); *hypoxia* (Ingalls et al. 1950, Murakami et al. 1962); *antitumour substances* (Haskin 1948, Murphy 1960, Endo & Abe 1965, Chaube & Murphy 1968, Singh & Sanyal 1972); *enzyme inhibitors* (Layton & Hallesy 1965, Wilson et al. 1968, Scott 1970, Layton 1971); *alkaloids* (Courtney & Valerio 1968, Joneja & Ungthavorn 1969); *anti-histamines* (King et al. 1965, Wilk 1969); *thalidomide* (Fabro & Smith 1966, Nudleman & Travill 1971). The role of mesoderm vs its covering ectoderm in a growing limb bud has been investigated extensively to analyse the mechanism of limb morphogenesis (Harrison 1918, Detwiler 1929, Saunders 1948, Zwilling 1956, Goetinck 1964). Tschumi (1954) suggested that digits which differentiate first use up about the usual amount of mesoderm, consequently leaving an inadequate amount for the digits which differentiate later, in the event of mesenchymal insufficiency. The order of mesenchymal condensation of digits in the hind limbs of *xenopus laevis* was found by Tschumi (1954, 1957) to be 4, 3, 5, 2 and 1. Forsthoefel (1963) found the order of differentiation of the toes of the mouse as 4, 5, 3, 2 and 1. Nogami (1964), while studying the frequency of malformations of the various digits of mice foetuses, induced by X-radiation, found digit 4 as the most resistant and hence least involved. No systematic study of the digital morphogenesis in rat foetuses seems to have been reported. The

present paper communicates the digital anomalies in rat foetuses induced by cyclophosphamide (an alkylating agent widely used clinically) given to pregnant rats during the 12th to the 16th day of gestation.

MATERIALS AND METHODS

Wistar female rats weighing about 200 g obtained from the Institute for Research in Reproduction, Bombay, were kept under standard conditions and mated in oestrus with the males of the same strain by placing them overnight in individual cages. The following day was counted as day zero of the pregnancy if sperms were found in the vaginal smears. Freshly prepared solution of the cyclophosphamide (Endoxan-Asta, manufactured by Khandelwal Laboratories Private Ltd., Bombay) in normal saline was administered by single intraperitoneal injection (I.P.) to pregnant rats on days 12-16 of gestation (Table 1). Dosage of the drug was worked out on the basis of mg/kg of the body weight of the pregnant animal at the time of the injection. Control rats received the same volume of the saline without the drug, during corresponding gestation periods.

All females were killed on day 21 of gestation; the abdominal wall was opened and both uterine horns were exteriorised. The number and position of live, dead and resorbed foetuses were noted. Surviving foetuses were removed after uterotomy, examined for gross malformations and preserved in fixatives. Hands and feet were later rechecked under the binocular dissecting microscope and details of various digital anomalies recorded. The gestation period in a few experimental rats was prolonged for 2 days by injections of progesterone (on the lines of Barrow & Rowland 1969) to see if with further development, the digital anomalies became more conspicuous.

Table 1. Incidence of malformations of hands and feet in the rat foetuses following maternal administration of cyclophosphamide on different days of gestation.

Days of treatment	Dose/kg	No. of litters	No. of implants	No. of resorptions	No. of hands examined	Abnormal hands (%)	No. of feet examined	Abnormal feet (%)
12th	8-15 mg	22	181	94	168	*137 (81.5%)	168	17 (10.1%)
13th	10-18 mg	19	164	11	300	227 (75.6%)	300	276 (92.0%)
14th	12-20 mg	24	202	10	380	§9 (2.3%)	380	48 (12.6%)
15th	15-20 mg	14	92	-	184	-	184	-
16th	20-30 mg	10	88	1	174	-	174	-
Control	Saline	16	130	-	260	-	260	-
Total		105	857	116	1466	373 (25.4%)	1466	341 (23.2%)

* $P < 0.001$ when compared with those of feet in the 12th day group.

§ $P < 0.001$ when compared with those of feet in the 14th day group.

RESULTS

As seen from Table 1, the hands and feet showing abnormal digits were found only in the litters which received cyclophosphamide injections during the 12th to 14th days of gestation. The foetuses from the control groups as well as from day 15 and 16 treated groups showed no digital abnormality. The thirteenth day of gestation turned out to be the most sensitive period as the teratogenic assault on this day resulted in 76 per cent of abnormal hands and 92 per cent of abnormal feet (Figure 1). Although hands were significantly more affected than feet by treatment on the 12th day (i.e. 82 per cent vs 10 per cent, $P < 0.001$), intervention on the 14th day resulted in reverse effect, i.e. 2 per cent abnormal hands and 13 per cent abnormal feet ($P < 0.001$). Higher dosage of the drug on any particular day did not prove more suppressive for the developing digits in that group. However, higher doses proved more lethal as reported in our earlier studies (Singh 1971, Singh & Sanyal 1972). Although the affected hands and feet of the right and left sides did not show perfect symmetry, the side differences were insignificant ($P > 0.70$ and $P > 0.90$).

The various types of digital anomalies (Figure 6), i.e. adactyly (absence of all digits), ectrodactyly (absence of one or more digits), syndactyly (fusion of digits) and brachydactyly (hypoplasia of digits) were found more frequently in the day 13 group than in other groups. These anomalies were significantly more common in the hands than in the feet in the day 12 group ($P < 0.001$; Figures 2 and 3) but became

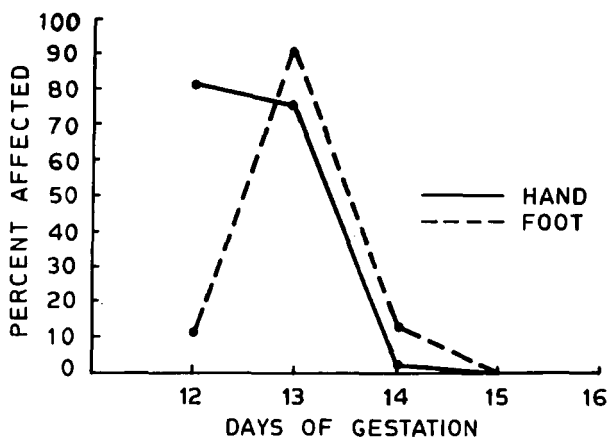


Figure 1. Hands and feet of rat foetuses having digital anomalies induced by maternal administration of cyclophosphamide on different days of gestation.

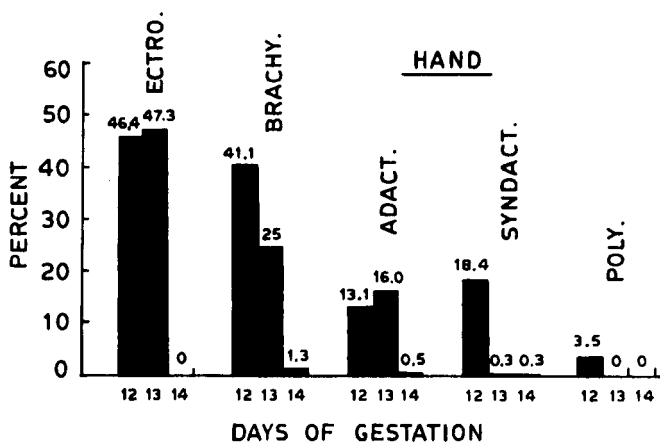


Figure 2. Various types of digital malformations in hands of rat fetuses induced by maternal administration of cyclophosphamide on different days of gestation.

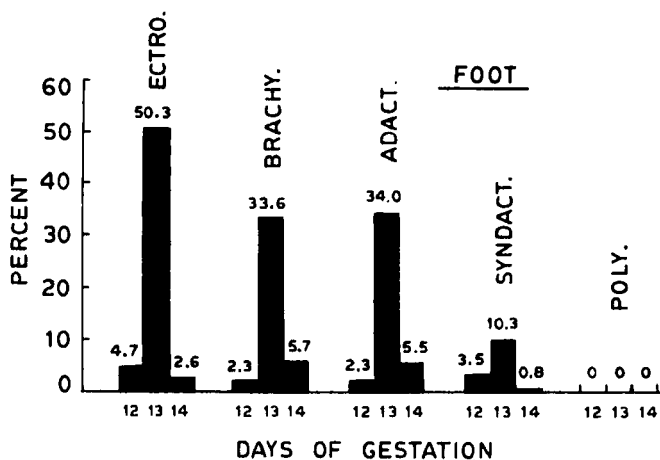


Figure 3. Various types of digital malformations in feet of rat fetuses induced by maternal administration of cyclophosphamide on different days of gestation.

predominant in the feet in the group treated on the 13th day of gestation ($P < 0.05$ to $P < 0.001$). In the day 14 group, brachydactyly and adactyly were significantly more common in the feet ($P < 0.01$ and $P < 0.001$) but the increase in the incidence of ectrodactyly and syndactyly was not significant ($P > 0.30$ and $P > 0.95$). Polydactyly (extra digit) was, however, found in the hands only and that also was confined to the group treated on the 12th day of gestation (Figure 2). All such hands showing polydactyly also exhibited syndactyly of some

digits (Figure 6 B). Pedunculated digit was occasionally seen in the toes in the day 13 group (Figure 6 H). Syndactyly in the hands was also practically confined to the day 12 group (Figure 2) and was more frequently seen than that of the feet (Figure 3) in this group (18 per cent vs 4 per cent, $P < 0.001$). Ectrodactyly was the commonest anomaly observed in the hands and feet and its incidence was a maximum in the day 13 group (47 per cent and 50 per cent respectively—Figures 2 and 3). Hypoplasia of the digits next in order of frequency was also more common in the day 13 group when digits of hands and feet were considered together (25 per cent and 34 per cent in the hands and feet respectively). No ectrodactyly was seen in any hands of the day 14 group whereas the feet showed it in 3 per cent of cases. However, in the day 12 group, ectrodactyly was more common in the hands than in the feet (46 per cent vs 5 per cent $P < 0.001$; Figures 2 and 3). All kinds of digital deformities were found to be more common in hands than in feet in the day 12 group (the incidence varying from 4 per cent to 46 per cent), while in the day 14 group the incidence of various types of digital anomalies of the hands as well as feet was markedly reduced (varying from 0 per cent to 6 per cent). Different types of anomalies existed in conjunction with each other in the affected hands and feet and manifested themselves in various transitional forms. Sometimes only rudimentary nodules (1–3) were seen at the distal end of the limbs (Figures 6 I and J).

Involvement of the individual digits in the hands and feet in the various groups are shown in Figures 4 and 5. As at times it was difficult to identify the individual digits without proper skeletal visualization, especially when only one digit was present, the findings were checked by the double blind method. Both in the hands and the feet, the third digit was found to be least commonly affected ($P < 0.01$ and $P < 0.001$ respectively) whereas the first and the fifth digits were most commonly involved (Figure 5). Taking the missing and rudimentary digits together, the suppression of digits occurred in the order of 1, 5, 2, 4, 3 digits in the hands and 1, 5, 4, 2, 3 digits in the feet (Figure 4). Fusion of the digits was most commonly observed between the second and the third digits both in the hands and the feet. The first digit was not fused with the second digit in any hand or foot. This pattern of individual digital involvement was observed to be more or less the same in all the three groups showing digital deformities.

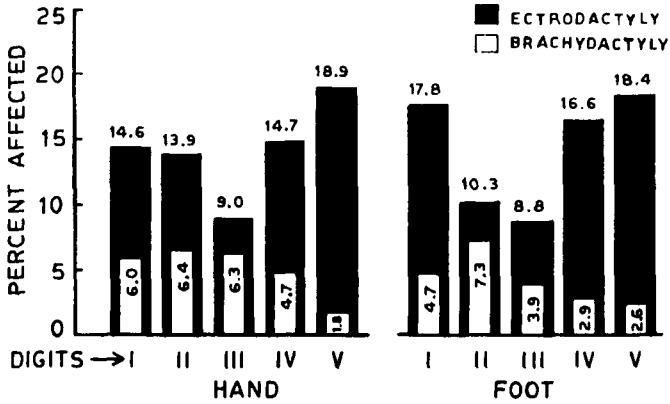


Figure 4. Incidence of ectrodactyly and brachydactyly in individual digits of rat fetuses induced by single I.P. injection of cyclophosphamide given to pregnant rats on the 12th-14th day of gestation.

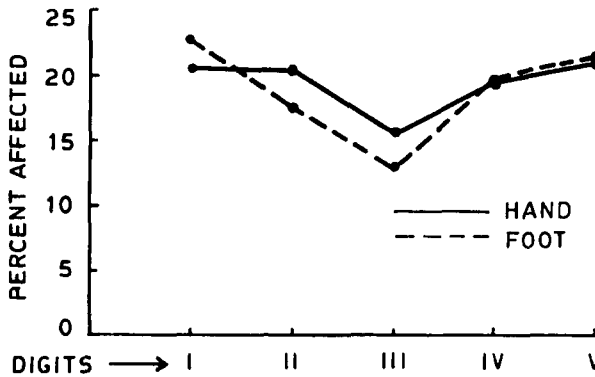
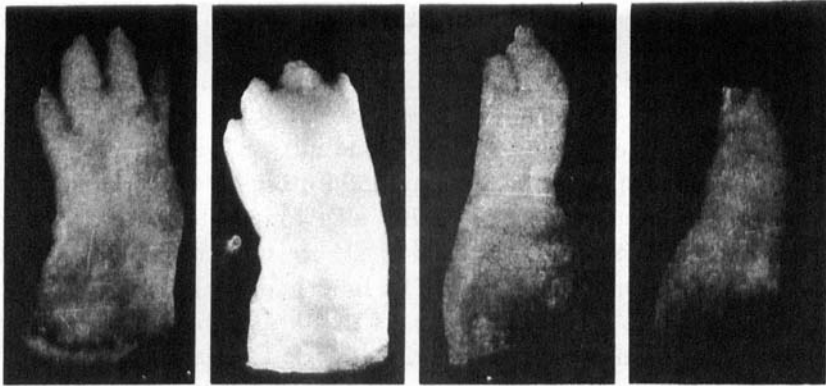


Figure 5. Susceptibility of individual digits of rat fetuses to the teratogenic action of single I.P. injection of cyclophosphamide given to pregnant rats on the 12th-14th day of gestation.

Figure 6. Involvement of the digits in 21-day-old rat fetuses after single I.P. injection of cyclophosphamide given to pregnant rats on the 12th-14th day of gestation. A. (Control) Hand (Lt.) normal (thumb not visible); B. (8 mg/12th day). Hand (Lt.) showing syndactyly and polydactyly (thumb not visible); C. (8 mg/12th day). Hand showing ectrodactyly and brachydactyly; D. (15 mg/13th day). Hand showing adactyly; E. (Control) Foot normal (Rt.); F. (15 mg/13th day). Foot showing ectrodactyly and brachydactyly and syndactyly; G. (15 mg/13th day) Foot showing ectrodactyly, brachydactyly and a rudimentary nodule; H. (15 mg/13th day). Foot showing ectrodactyly and pedunculated and deviated great toe; I. (15 mg/13th day). Hand showing ectrodactyly, brachydactyly, rudimentary nodule, (Palmar view), J. (15 mg/13th day). Foot showing ectrodactyly, brachydactyly and rudimentary nodule (Planter view); K. (12 mg/12th day). Foot of 23-day-old foetus (progester one treated) showing ectrodactyly and syndactyly; L. (15 mg/13th day) 21-day-old foetus showing digital anomalies of hands and feet besides other anomalies.

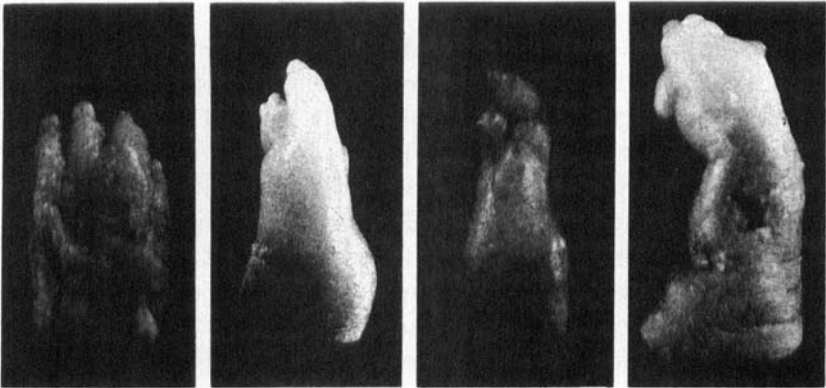


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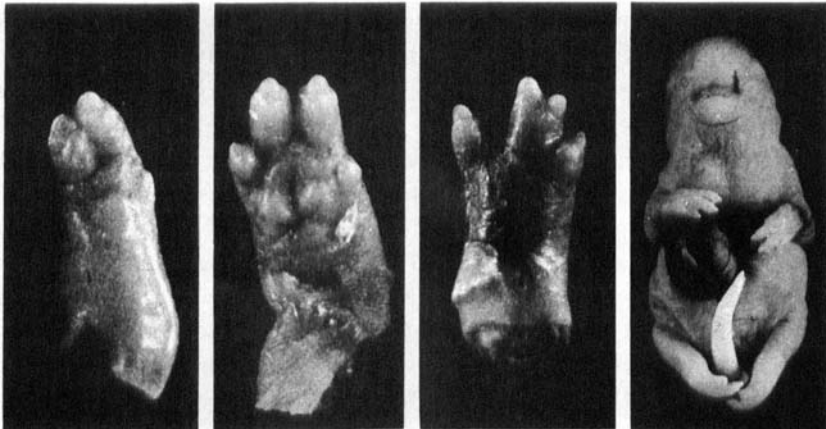


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I

J

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L

DISCUSSION

While studying digital anomalies of mouse fetuses induced by X-radiation, Nogami (1964) found the 12th day to be the most sensitive period, which corresponded with the findings of Russell (1950, 1954) who had observed a similar critical period for digital anomalies in mice. Neifach (1960) and Gebhardt (1970) also found the period between the 11th and 12th day of gestation to be more sensitive for the production of digital anomalies in mice. The limb bud of the rat lags behind that of mice by a period of $1\frac{1}{2}$ days in its appearance and further differentiation (Ihara 1970). The critical period when developing digits in rat fetuses are most vulnerable to any teratogenic assault as observed in the present study (i.e. 13th day) is thus identical with that observed in mice. Since all parts of the embryo are not fashioned simultaneously but emerge in their due succession and order, under the influence of organisers, the brunt of the disturbance is borne chiefly by the tissue or organ which is undergoing a rapid phase of development at the time when such an interference occurs. In fact each developing organ or part passes through an individual critical period during which it is undergoing accelerated growth and differentiation, and manifests marked susceptibility to the injurious influences brought to bear on it. Other parts may or may not be so scheduled as to be sensitive at that particular moment to those particular influences. Since the forelimb buds appear a day earlier than the hind limb buds in rats (Edwards 1968) as well as in mice (Rugh 1968), intervention during early gestation period (12th day) affected digital anomalies of the hand more than those of the feet, as the latter were not passing through their critical stages of proliferation and differentiation at the time. This occurred about a day later, i.e. on the 13th day, when maximum digital anomalies of feet resulted. A similar sequence of digital involvement has been reported by Kreybig (1968) and Gebhardt (1970). As the critical phase of differentiation of the digits was over, administration of the drug on the 15th and 16th days of gestation did not induce any digital anomaly (Table 1). However, development of anomalies is not only dependent on the critical stage as was thought earlier (Stockard 1921, Asling et al. 1955) but the tissues also show differential susceptibility according to the agent employed (Nishimura 1959). Why, for example, does the thalidomide drug pick up only the mesenchymal tissue and cortisone induces mainly facial anomalies while rubella infection and other teratogenic agents have no similar effect, when all

are acting in the same gestation period (Cahen 1964)? The results of teratogenicity further depend not only on the species of the animals used but also on the strain of the animal. Nogami (1964) found the digital malformations varying in total number and even in kind in two strains of mouse fetuses treated by X-radiation under similar experimental conditions. However, the critical period of vulnerability and the type of digital malformations induced by cyclophosphamide in rats in the present study closely resemble those of mice caused by X-irradiation. The X-irradiation probably causes a cellular necrosis of the digital primordia (Murakami et al. 1963). Cyclophosphamide (a radiomimetic drug) is first activated in the liver of the host, resulting in liberation of its alkylating radical (Brock 1967) which is highly reactive and its major action seems to be related to the inhibition of deoxyribonucleic acid (DNA). The prolonged inhibition of DNA synthesis may lead to localised cell death sufficient to upset proliferative rates within the organs, resulting in malformations (Ritter et al. 1971). The teratogenic actions of cyclophosphamide (Chaube et al. 1967, Singh 1971), nitrogen mustard (the parent alkylating agent—Haskin 1948) and X-radiation (Russell 1954, Nogami 1964) have shown striking similarities, possibly because of their similar mode of action, i.e. inhibition of DNA synthesis. The brunt of the damage caused by these agents is, however, borne mainly by the mesenchymal tissue (Hicks et al. 1957, Murphy et al. 1958, Neifach 1960, Greenberg & Tanaka 1964, Singh et al. 1971).

The various digital anomalies (adactyly, ectrodactyly, syndactyly, brachydactyly, etc.) are manifestations of the injurious effect of the teratogen on the proliferating and differentiating mesenchymal tissue in the regions of hands and feet. Nogami (1964) found that a higher dose of X-radiation resulted in an increased number of missing digits (ectrodactyly), but the incidence of syndactyly and brachydactyly was correspondingly decreased. Lower doses of X-rays produced the reverse effect (i.e. less of ectrodactyly and more of syndactyly and brachydactyly). Our findings do not fall in line with those of Nogami (1964) because higher doses of cyclophosphamide were usually not found to be more suppressive to the developing digits. However, the higher doses proved more lethal as reported in our earlier studies (Singh 1971, Singh & Sanyal 1972). The embryo-lethal effect is known to be separate from the teratogenic effect (Roussel & Tuchmann-Duplessis 1968, Chamberlain & Goldyne 1970, Gebhardt 1970). Absence of a digit either individually (ectrodactyly) or along with all other digits (adactyly) was

the commonest type of anomaly seen amongst the malformed hands and feet. The antimitotic activity of cyclophosphamide led to a diminished amount of the mesenchyme which was not sufficient for the differentiation of all the digits thus resulting in absence of one or more digits. In such mesenchymal insufficiency the digits which differentiate first, use up the normal amount of mesenchyme leaving little or none for the digits differentiating relatively later (Tschumi 1954). If the amount of mesenchyme left is small, the digits differentiating later may remain united (syndactyly) and if no mesenchyme is left these may not differentiate at all and disappear (ectrodactyly). Polydactyly occurred only in the hands (3.5 per cent) and in the group treated on 12th day alone. All such cases showed syndactyly also (Figure 6). Nogami (1964) found polydactyly in mice occurring only in the feet and it appeared a little earlier than the syndactyly which was not associated with it. The critical stage for the induction of polydactyly has been found to precede the one for other digital anomalies, not only in mammals (Gebhardt 1970), but also in amphibia (Gebhardt & Faber 1966). No satisfactory explanation, however, seems available for this phenomenon.

The order of mesenchymal condensation of digits in the hind limbs of *xenopus laevis* was found by Tschumi (1954, 1957) to be 4, 3, 5, 2, 1 and Forsthoefel (1963) found the order of differentiation of the toes of the mouse as 4, 5, 3, 2, 1. As the digits on the preaxial border, i.e. 2 & 1, differentiate last of all, these are commonly affected, resulting in syndactyly or ectrodactyly. The digit 4 which is thus first to differentiate has been found to be usually most resistant to the teratological insults (Nogami 1964, Muto 1971). However, Maeda (1970) did not find any consistency in such a resistance because it varied with the stage of development of the digits when the teratogen was administered. In the hands, he found the digit 2 was most resistant when treatment was given on the 10th day of gestation, but in the day 11 group digit 1 was least involved. On the 12th day, digit 4 was least affected while on the 13th day, digit 2 was equally resistant. In our present study digit 3 was found to be least involved in the hands ($P < 0.01$) as well as in the feet ($P < 0.001$). The order of suppression of digits was noted as 1, 5, 2, 4, 3 in the hands and 1, 5, 4, 2, 3 in the feet. Nogami (1964) also found a different order of suppression in the fingers and toes of mice. This is also in line with the findings of Maeda (1970) who found digits 1 and 5 to be more commonly affected both in the hands and feet in the group of rats treated on the 13th day of gestation. According

to Grüneberg (1961) the blastema for digits 2 to 4 seem to be laid down first and both marginal rays follow later. This may account for the higher frequency of involvement of digits 1 and 5. Muto (1970) also observed digit 5 of the forelimb of toad larva to be most commonly involved in teratological experiments and found it difficult to explain on the hypothesis of Tschumi (1954). Further, brachydactylism may not be due to shortening of the metatarsals but may result from hypophalangism alone (Muto 1969). The phalanges of digit 3 were found to be most resistant by Muto (1971) which could explain the least involvement of digit 3 in our study. Systematic study of the order of blastemal condensation of various digits in rats and their order of chondrification and ossification may throw more light on the morphogenesis of digits.

SUMMARY

Single intraperitoneal injections of cyclophosphamide (endoxan-asta) administered to pregnant rats on the 12th–14th day of gestation resulted in a variety of malformations of the digits of the fetuses collected on day 21. No digital anomaly was detected in the control group or in the groups treated on the 15th and 16th days of gestation. Dose response relation could not be established for any type of digital malformation on any day of treatment. Digits of the hands were most frequently involved in the day 12 group (82 per cent) as compared to those of feet (10 per cent). Treatment on the 14th day of gestation affected more toes (13 per cent) than fingers (2 per cent). The thirteenth day of gestation was found most susceptible for induction of digital anomalies and it affected 92 per cent of feet and 76 per cent of hands. Polydactyly occurred exclusively in the hands and only in the 12 day group. Syndactyly most commonly affected the digits 2 and 3 both in the hands and feet. Ectrodactyly and brachydactyly, the commonest digital anomalies mainly involved digits 1 and 5 in both limbs. The antimitotic action of cyclophosphamide mostly suppressed the mesenchymal blastema destined for the marginal digits (1 and 5) resulting in hypoplasia or disappearance of these digits. Digit 3 was found to be most resistant to its teratogenic effects.

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