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Pattern of Limb Malformations in Mice Induced by Methoxyacetic Acid

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ABSTRACT

The present study investigated the pattern of limb malformations induced in mice by methoxyacetic acid (MAA), one of di(2-methoxyethyl) phthalate (DMEP) metabolites. Pregnant Jcl:ICR mice were given orally at gestational day (gd) 10.5, 11.0, or 11.5 (vaginal plug = gd 0) a single dose of MAA 10 mmol/kg of body weight. Fetuses were examined at gd 15.5 for external and skeletal malformations. Limb defects were maximum in frequency and severity after administration at gd 11.5. Forelimbs had greater susceptibility than hindlimbs. Treatment at gd 10.5 produced cutaneous or osseous syndactyly between digits I and II, II and III, and ectrodactyly of digit II or digit I. Intercalary defects in the forelimbs were also detected. No hindlimb malformations were induced. Treatment at gd 11.0 induced ectrodactyly of digit II and digit I in the forelimbs, as well as osseous syndactyly or intercalary defects of the metacarpals or phalanges, and the frequency became increased. Hindlimb malformations were also detected in a small number with syndactyly between digits I and II or ectrodactyly in digit I. Treatment at gd 11.5 induced ectrodactyly in the forelimbs. Half of the forelimbs showed ectrodactyly with four missing digits (digits I, II, III, and V) and the remaining limbs showed ectrodactyly with a similar frequency with one, two, or three missing digits, either in the pre- or postaxial area. In the hindlimbs ectrodactyly with one, two, or three missing digits was the most common malformation observed. Syndactyly between digits I and II was also induced in a small percentage.

Key words: *Teratogenesis, Plasticizers, Syndactyly, Ectrodactyly*

Phthalate esters are industrial chemicals which are commonly used as plasticizers in the manufacture of flexible plastics. Due to wide spread distribution, they are becoming ubiquitous environmental pollutants[4,9], hence there have been concerns regarding their toxicity. Di(2-methoxyethyl) phthalate (DMEP), is one of the most toxic and potent teratogens among phthalate esters in rats[15,19]. Previous investigation[2] indicated that 2-methoxyethanol (2-ME), derived by metabolism of DMEP, was embryotoxic and teratogenic in rats. A rat teratology study[16] revealed that DMEP was hydrolyzed in vivo to 2-ME and oxidized to methoxyacetic acid (MAA) , the proximate teratogen. The teratogenic effects of 2-ME were studied in rats[6,14,23] and mice[5,6,7,13] and in non-human primates[17]. Embryotoxicity and teratogenicity of MAA were also studied in rats[1,12,23]. Previous studies in our laboratory (unpublished observation) showed that MAA was embryotoxic and induced various limb malformations in mice.

The purpose of this paper is to describe the pattern of limb malformations in mice induced by MAA given at various stages of development.

MATERIALS AND METHODS

Colony-bred Jcl:ICR mice from CLEA Japan, Inc. were used in this experiment. Mature females were placed with males overnight. Copulation was ascertained by the presence of a vaginal plug on the following morning, and 0:00 A.M. of that day was denoted as the start of day 0 of gestation (gd 0).

Pregnant mice were given a single oral dose of MAA (supplied by Katayama Chemical, Japan) at 10 mmol/kg of body weight dissolved in distilled water, at gd 10.5, 11.0 or 11.5. This dose is equivalent to 0.8 ml/kg. The dose employed was chosen on the bases of teratologic studies conducted in our laboratory. This dose was not maternally toxic, and surviving fetuses had various limb malformations at high frequencies. Control animals received the same volume of vehicle at the corresponding time points.

For the description of gross malformations, the pregnancy was terminated on gd 15.5 by cervical dislocation. Following Caesarean section, implants

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were counted in situ, and the number of dead or resorbed implants was determined. Living fetuses were removed, weighed, and examined under a dissecting microscope for external malformations, especially limb deformities. For skeletal observations, fetuses were fixed in 95% ethanol and processed for cartilage staining with Alcian blue according to the method of Inouye[8] with slight modification. The data obtained for fetal weight, number of live fetuses, and malformed limbs were statistically analyzed on a per-litter basis by Student's t-test.

RESULTS

Table 1 summarizes the embryotoxic and teratogenic data. Fig. 1 shows the frequency of limb malformations according to the day of administration. Figs. 2, 3, and 4 show examples of external and skeletal anomalies in forelimbs from fetuses treated at gd 10.5, 11.0, and 11.5, respectively. Fig. 5 shows anomalies in hindlimbs treated at gd 11.5.

Table 1. Embryotoxic and teratogenic effects of MAA in mice with a single oral dose of 10 mmol/kg at gd 10.5, 11.0, or 11.5

	GESTATIONAL DAY OF TREATMENT					
	10.5		11.0		11.5	
	Control	Treated	Control	Treated	Control	Treated
No. dams	10	13	10	11	7	10
No. implants	137	178	132	147	103	140
( ): Mean ± SD	(13.7 ± 2.1)	(13.7 ± 1.9)	(13.2 ± 2.1)	(13.6 ± 1.8)	(14.7 ± 2.9)	(14.0 ± 2.2)
No. dead	2	48*	2	9	3	11
( ): %	(1.5)	(27.0)	(1.5)	(6.1)	(2.9)	(7.9)
No. live	135	130	130	138	100	129
( ): Mean ± SD	(13.5 ± 2.1)	(10.0 ± 5.8)	(13.0 ± 2.5)	(12.5 ± 2.5)	(14.3 ± 2.6)	(12.9 ± 2.5)
Fetal weight: g	0.46 ± 0.04	0.35 ± 0.08**	0.47 ± 0.04	0.35 ± 0.06**	0.45 ± 0.05	0.34 ± 0.06**
% Limb anomalies						
Forelimb	0	80**	0	96**	0	100**
Hindlimb	0	0	0	25*	0	94**

Data were calculated on the basis of the litter as a sample unit.
* Significantly different from controls, $p<0.05$
** Significantly different from controls, $p<0.005$

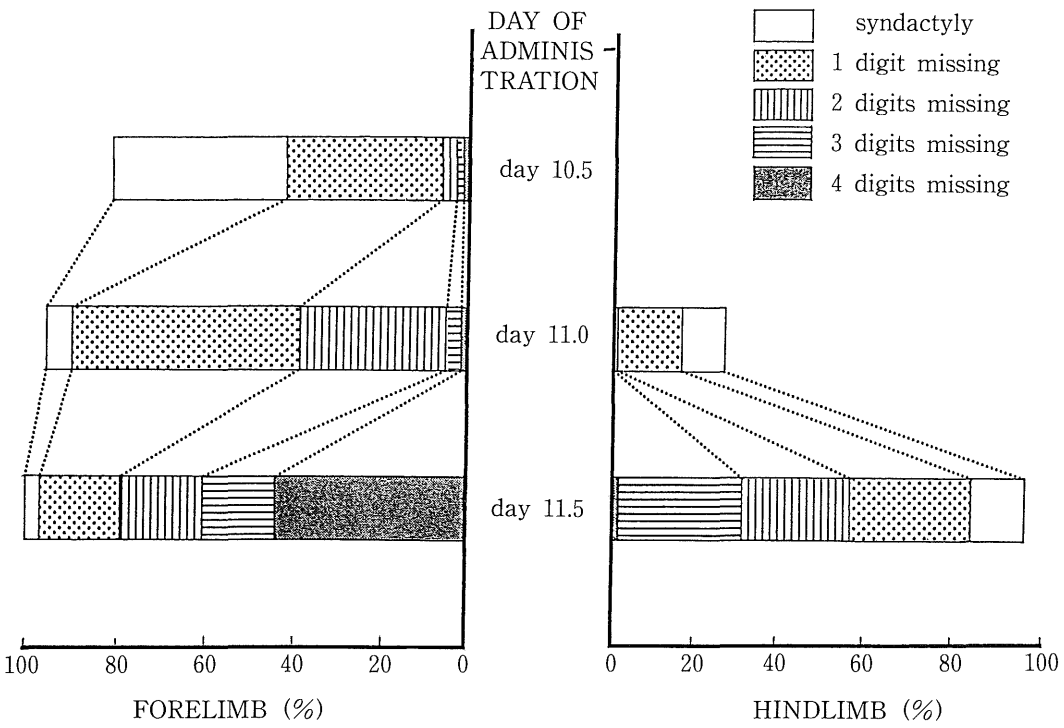

Fig. 1. Incidence of limb malformations in mouse fetuses from dams treated with an oral dose of MAA 10 mmol/kg. Observation at gd 15.5.

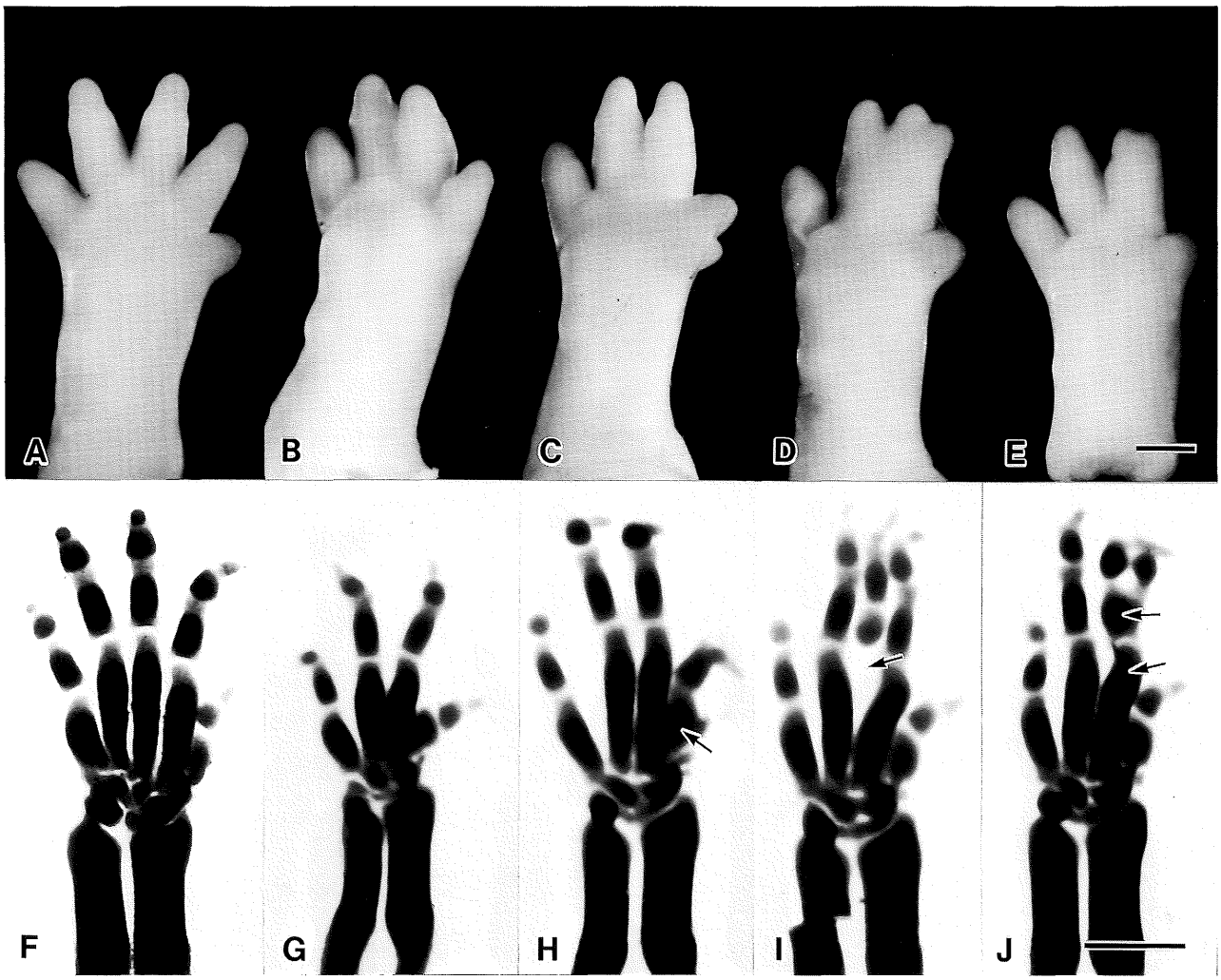

Fig. 2. External (A - E) and skeletal (F - J) features of forelimbs at gd 15.5. Oral administration at gd 10.5.
A, F: Normal forelimbs. B - E, G - J: Forelimbs treated with MAA.
B, G: Ectrodactyly with missing digit II.
C, H: Osseous syndactyly between MC I-II (arrow).
D, I: Syndactyly involving digits I, II and III with an intercalary defect of MC III (arrow).
E, J: Osseous syndactyly between digits II - III, including MC and phalanges (arrows).
A - E, and F - J are at the same magnification, respectively.
Scale bars = 0.5 mm.

External observations

Malformations of limbs induced by MAA were more frequent in forelimbs than hindlimbs, especially in the groups treated at gd 10.5 and 11.0. In the group treated at gd 10.5, only forelimb malformations were observed. In the group treated at gd 11.0, forelimb malformations were about four times more frequent than hindlimb malformations, and in the group treated at gd 11.5, malformations were observed equally in forelimbs and hindlimbs, and involved nearly all fetuses. In the group treated at gd 10.5, syndactyly was the most common anomaly. Fusion between digits I and II (Fig. 2 C) was frequent, followed by that between digits II and III (Fig. 2 E), and that between other digits was infrequent. Ectrodactyly with one missing digit (Fig. 2 B) was also observed in a high incidence. In the group treated at gd 11.0, the incidence of ectrodactyly with one or two missing digits (Fig. 3 B, C) increased, and ectrodactyly with three or four missing digits (Fig. 3 D, E) was also observed in small percentages and the incidence of syndactyly markedly decreased. In the hindlimbs of the group treated at gd 11.0, syndactyly between digits I and II or between digits II and III, and ectrodactyly with one missing digit were found. The pattern was similar to that observed in the forelimbs of the group treated at gd 10.5. In the group treated at gd 11.5, ectrodactyly with four missing digits (Fig. 4 E) was observed in half of the forelimbs and ectrodactyly with one, two, or three digits missing (Fig. 4 B, C, D) was also detected with the same frequency. In the hindlimbs, ectrodactyly with one, two, or three missing digits (Fig. 5 B, C, D) was the most common malformation, and syndactyly between digits I and II, and between other digits was

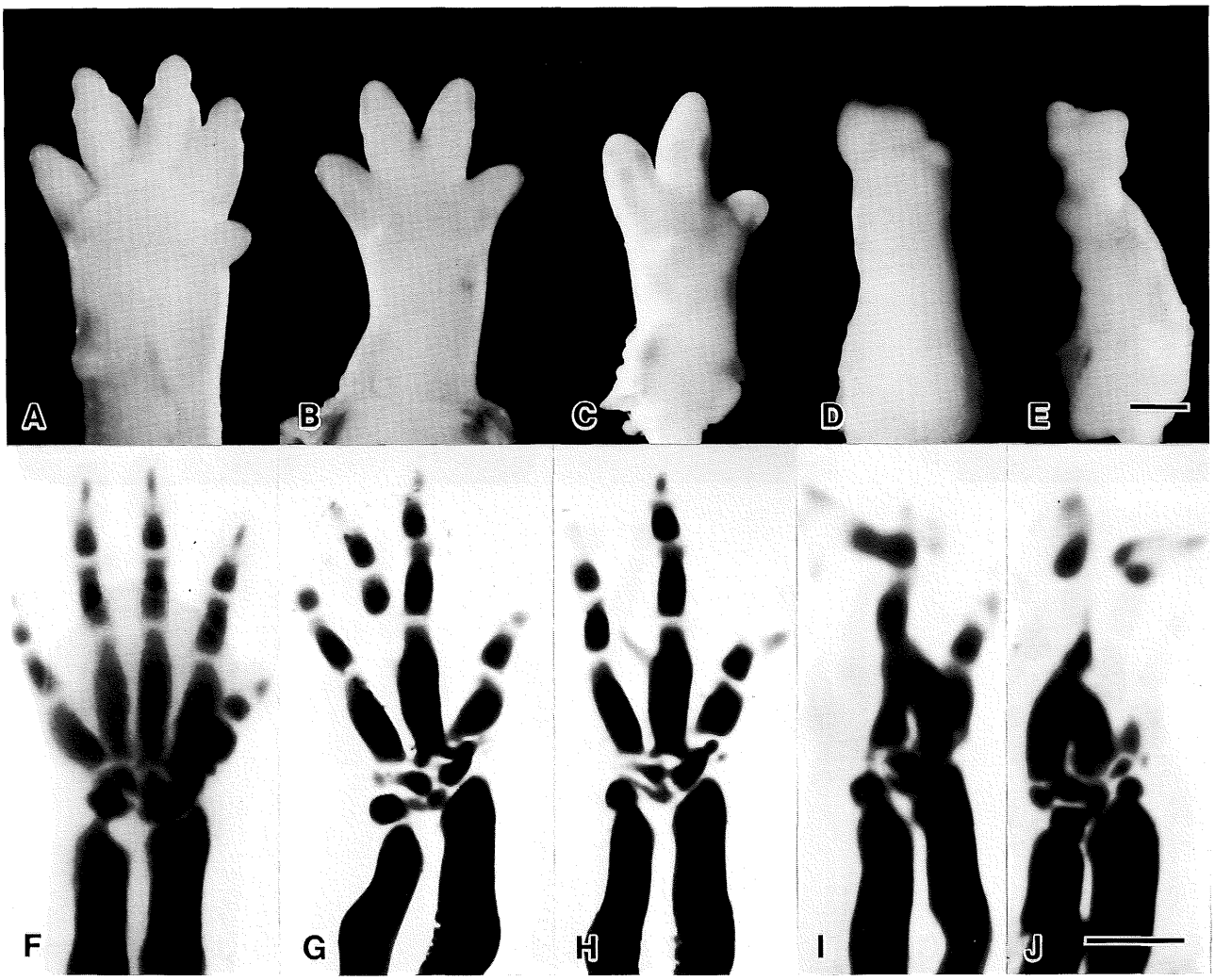

Fig. 3. External (A - E) and skeletal (F - J) features of forelimbs at gd 15.5. Oral administration at gd 11.0.
A, F: Normal forelimbs. B - E, G - J: Forelimbs treated with MAA.
B, G: Ectrodactyly with an intercalary defect of MC IV and missing digit II.
C, H: Ectrodactyly with missing digits II and IV.
D - E, I - J: Ectrodactyly and osseous syndactyly with intercalary defect with two and three missing digits.
A - E, and F - J are at the same magnification, respectively.
Scale bars = 0.5 mm.

also found.

Skeletal observations

In the group treated at gd 10.5 (Fig. 2), the most common forelimb malformation was ectrodactyly of digit II (Fig. 2 G). Ectrodactyly of digit I was also found in a small number. Osseous syndactyly between metacarpals (MC) I and II (Fig. 2 H) was frequently observed. Osseous syndactyly between other MC and/or phalanges (Fig. 2 J) was less frequently observed. Intercalary defects of MC (Fig. 2 I) or phalanges in various degrees were found in a small number.

In the group treated at gd 11.0 (Fig. 3), the characteristic malformation was ectrodactyly with missing digit II (Fig. 3 G). Ectrodactyly of digits I had been induced in a small percentage. Ectrodactyly with two missing digits (Fig. 3 H) was more frequent. Ectrodactyly with syndactyly and intercalary defects (Fig. 3 I, J) were the prominent feature at this stage. In the hindlimbs, ectrodactyly with one missing digit in the preaxial area, or osseous syndactyly between MC I and II was observed.

In the group treated at gd 11.5 (Fig. 4), the most common forelimb malformation was ectrodactyly with four missing digits (digits I, II, III and V) (Fig. 4 J), or ectrodactyly with one, two, or three missing digits in the preaxial or postaxial area (Fig. 4 G, H, I). In the hindlimbs, ectrodactyly with one, two, or three missing digits in the preaxial and/or postaxial area (Fig. 5 F, G, H), and also some limbs with syndactyly were observed.

No abnormalities were observed in the long bones of the forelimb and hindlimb.

DISCUSSION

Teratogenic studies of DMEP and its metabolites have been performed in rats, mice and rabbits. In

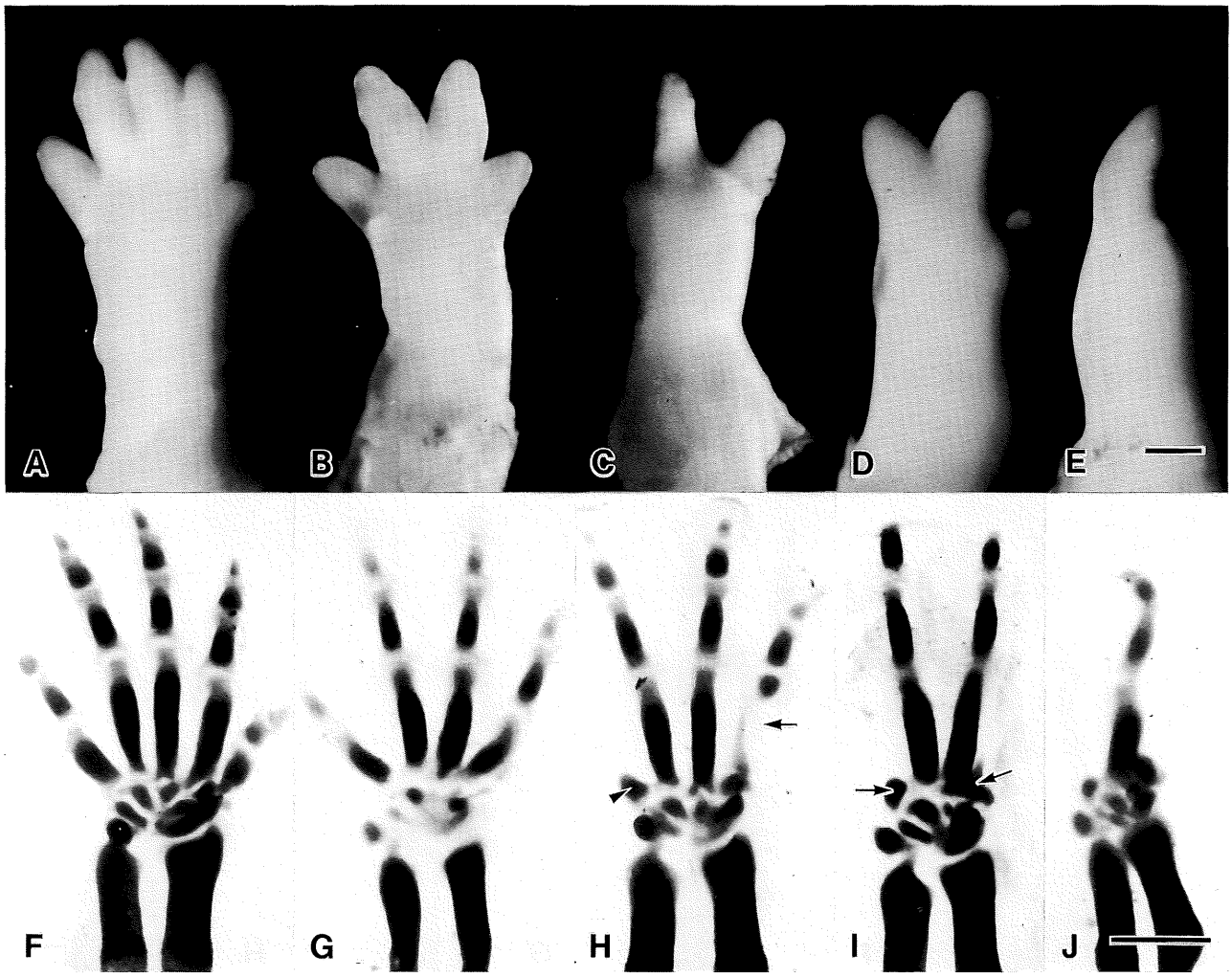

Fig. 4. External (A - E) and skeletal (F - J) features of forelimbs at gd 15.5. Oral administration at gd 11.5.
A, F: Normal forelimbs. B - E, G - J: Forelimbs treated with MAA.
B, G: Ectrodactyly with missing digit I.
C, H: Ectrodactyly with missing digits I and V. Intercalary defect of digit II (arrow) and a remnant of MC V (arrowhead).
D, I: Ectrodactyly with missing digits I, II and V. Remnants of MC I and MC V (arrows).
E: Ectrodactyly with missing digits I, II, III and V.
A - E, and F - J are at the same magnification, respectively.
Scale bars = 0.5 mm.

Wistar rats, DMEP and its metabolites given on gd 12 produced hydronephrosis, heart defects, and short limbs or tails, and ventral polydactyly[16]. Hanley et al[6] exposed pregnant rats, mice, and rabbits to 2-ME vapor during organogenesis. Limb defects were induced in rabbits. When given on gd 12 intraperitoneally to Wistar rats, 2-ME induced forelimb and hindlimb malformations[18]. Ectrodactyly, syndactyly or radioulnar shortening, and ventral duplication of hindlimb digits were observed. Teratogenic effects of 2-ME were observed in mice when it was given orally during organogenesis[7]. Syndactyly, oligodactyly and stunted digit I were maximally induced after administration on gd 11. The preaxial phalanges were primarily affected. Brown et al[1] gave a single intraperitoneal injection of MAA into rats during organogenesis, and obtained various skeletal anomalies including absence or shortening of the ulna, radius and fibula. The present experiment was the first teratological study of MAA in mice. Our findings were generally comparable to those reported by others in other species or with other metabolites, excepting that we found neither ventral polydactyly nor radioulnar shortening. These differences may be partly due to species differences, and partly due to developmental stage differences.

Different types of reductional malformations of the digit can be produced by teratogens administered at different stages of gestation[20]. Limb malformations were prevalent when exposures to 2-ME occurred between gd 9 and 12 in mice[7]. Paw anomalies were maximal after administration on gd 11, and forepaws exhibited greater suscepti-

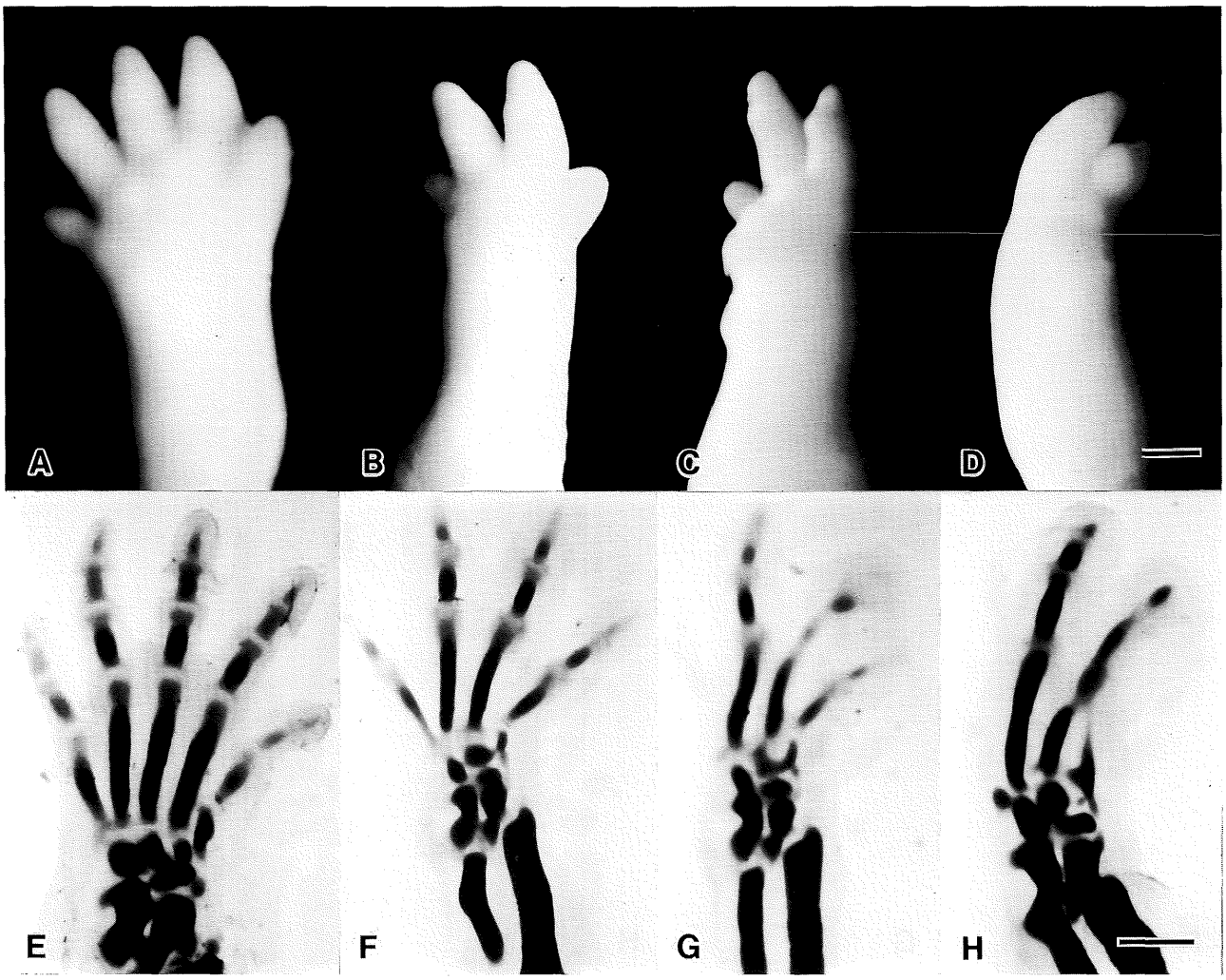

Fig. 5. External (A - D) and skeletal (E - H) features of hindlimbs at gd 15.5. Oral administration at gd 11.5.
A, E: Normal hindlimbs. B - D, F - H: Hindlimbs treated with MAA.
B, F: Ectrodactyly with missing digit I.
C, G: Ectrodactyly with missing digits I and V.
D, H: Ectrodactyly with missing digits I, II and V.
A - D, and E - H are at the same magnification, respectively.
Scale bars = 0.5 mm.

bility than hindpaws. These results with 2-ME were in accordance with our results with MAA, although the stages covered were narrower in our experiments. The similarities in the pattern of limb malformations in forelimbs treated at gd 11.0 and those in hindlimbs treated at gd 11.5 can be explained by the fact that the development of the hindlimb in mice is 12 to 24 hr behind that of the forelimb[10,11].

It is well known that there is agent specificity in the pattern of limb malformations induced by teratogens. For example, an excessive dose of vitamin A given at gd 10.5 to mice induced limb reduction deformities involving both proximal and distal components[21,22] whereas 5-fluorouracil given at gd 10.5 to mice produced polydactyly in the hindlimb without affecting the proximal components[3,22]. Under the present conditions, MAA induced only reduction deformities in the autopod, occasionally with intercalary defects. These characteristics may be explained by the strong cytotoxicity and rapid excretion of MAA, together with the regenerative capacity of the embryonic limb. For further elucidation of these characteristics, studies on the pathogenesis of MAA-induced limb malformations are warranted.

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