Table 4
Skeletal examinations in fetuses of rats given DTG on days 6–19 of pregnancy

Dose (mg/kg)	0 (control)	10	20	40
Total no. of fetuses (litters) examined	184(24)	176(24)	170(24)	130(20)
Total no. of fetuses (litters) with malformations	1	1	13(6)*	26(12)**
Split cartelige of thoracic centrum	0	0	1	1
Fused cartilage of cervical vertebral arches	0	1	1	1
Fused cartilage of ribs	1	0	0	0
Absence, fusion or malposition of caudal vertebrae	0	0	8(3)	10(8)**
Absence or fusion of phalanges	0	0	5(3)	18(9)**
Fusion of metacarpal/metatarsal and phalanx	0	0	0	2(2)
Absence or fusion of metacarpals	0	0	0	4(4)*
Shortening of tibia and fibula	0	0	0	1
Total no. of fetuses (litters) with variations	10(7)	16(9)	16(11)	12(8)
Bipartite ossification of thoracic centrum	0	2(1)	1	0
Dumbbell ossification of thoracic centrum	0	1	0	0
Unossified thoracic centrum	1	1	0	1
Variation of number of lumbar vertebrae	1	0	0	2(1)
Wavy ribs	0	1	1	0
Short supernumerary rib	9(6)	12(7)	14(10)	4(4)
Short 13th rib	0	0	0	2(2)
Sacralization of lumbar vertebra	0	0	0	2(1)
Bipartite ossification of sternebra	0	0	1	1
Asymmetry of sternebra	0	0	0	1
Degree of ossification[a]				
No. of sacral and caudal vertebrae	7.3 ± 0.5	7.5 ± 0.5	7.5 ± 0.5	7.0 ± 0.6*
No. of sternebrae	4.6 ± 0.4	4.8 ± 0.5	4.6 ± 0.4	4.2 ± 0.4*
No. of metatarsals	8.0 ± 0.0	7.9 ± 0.3	7.8 ± 0.4	6.7 ± 1.4*

[a] Values are given as the mean ± S.D.
* Significantly different from the control ($p < 0.05$).
** Significantly different from the control ($p < 0.01$).

characterize the effects of DTG on embryonic/fetal development. The findings of the present study confirmed the results of a previous screening study and extended the understanding of the reproductive and developmental toxicity of DTG. The present data showed that the prenatal oral administration of DTG produced maternal toxicity, as evidenced by deaths, neurobehavioral changes, decreased body weight gain and reduced food consumption, and developmental toxicity, as evidenced by a high incidence of postimplantation loss, a decreased number of live fetuses and lower weight of fetuses, and teratogenicity, as evidenced by a higher incidence of fetuses with external and skeletal malformations.

DTG is a specific sigma receptor ligand [3] and sigma receptor ligands can modulate neurotransmissions, including the noradrenergic, glutamatergic and dopaminergic system [10,21,22]. The systemic injection of DTG has been reported to cause neurobehavioral changes in rats [4,6,7,9,22]. The present study shows that the oral administration of DTG also induced neurobehavioral changes at 20 and 40 mg/kg bw/day in pregnant rats. Lowered body weight gain at 20 and 40 mg/kg bw/day and food consumption at 40 mg/kg bw/day were also observed in pregnant rats. These findings indicate that DTG is maternally toxic at 20 mg/kg bw/day and higher.

The sex ratio (males/females) was significantly lowered in all DTG-treated groups. The values for sex ratio were 0.429–0.521 in the background control data for the last 6 years in the laboratory performed present study. Statistically significant changes in the sex ratio observed in the present study were considered to be unrelated to the administration of DTG, because the values for sex ratio in the DTG-treated groups were within the range of the historical control data, no increased embryonic/fetal deaths were detected at 10 and 20 mg/kg bw/day and the control value for the sex ratio was very high in the present study. A decreased number of live fetuses, increased incidence of postimplantation loss, and reduced weights of fetuses and placentae were detected at 40 mg/kg bw/day. A decreased number of live fetuses and increased incidence of postimplantation loss indicate embryonic/fetal lethality, and reduced weights of fetuses and placentae indicate intrauterine growth retardation. These findings indicate that DTG is toxic to embryonic/fetal survival or fetal growth at 40 mg/kg bw/day when administered during the time of implantation to the term of pregnancy.

In our previous reproductive and developmental screening test [15], the total number of fetuses with external malformations, but not individual malformation, was significantly increased at 50 mg/kg. At this dose, oligodactyly and tail anomalies were frequently observed, and the teratogenic effect of DTG was strongly suggested. No malformed fetuses were found at 20 mg/kg bw/day in our previous study. In the present study, morphological examinations in the fetuses of exposed mothers revealed increased incidence of fetuses with external and skeletal malformations at 20 and 40 mg/kg bw/day.

Fetuses with external, internal and/or skeletal malformations and/or variations were found in all groups. The malformations and variations observed in the present study are of the types that occur spontaneously among the control rat fetuses [23–26]. At 40 mg/kg bw/day, significantly higher incidences of the total number of fetuses with external and skeletal malformations were detected, and significantly higher incidences of individual types of external and skeletal malformation were also noted. At 20 mg/kg bw/day, the incidence of the total number of fetuses with skeletal malformations was significantly higher than that of control group. Although the incidence of individual types of skeletal malformation was not significantly increased at 20 mg/kg bw/day, types of external and skeletal malformations observed at this dose were the same as those observed at 40 mg/kg bw/day. Consideration of the sum of these findings suggests that a conservative estimate of the LOAEL for the teratogenic dose of DTG is 20 mg/kg bw/day in rats when administered during the time of implantation to the term of pregnancy. DTG caused suppression of body weight gain and neurobehavioral changes in dams and abnormally morphological development and developmental delay in the offspring of rats at 20 and 40 mg/kg bw/day. Therefore, the teratogenic effects of DTG at doses without maternal toxicity, a selective teratogenicity of DTG, was not found in the current study. There are no available reports in which the developmental toxicity of DTG is assessed in any other animal species. Further studies are needed to confirm the reproductive and developmental toxicity of DTG in additional species. Developmental neurotoxicity and multigeneration studies are also required to support the conclusion of the prenatal hazard of DTG.

In conclusion, DTG caused maternal neurobehavioral changes and decreased body weight gain at 20 mg/kg bw/day and higher, embryonic/fetal deaths and lowered fetal weight at 40 mg/kg bw/day, and increased incidence of fetuses with malformations at 20 mg/kg bw/day and higher when administered during the time of implantation to the term of pregnancy in rats.

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Reproductive and developmental toxicity screening test of basic rubber accelerator, 1,3-di-*o*-tolylguanidine, in rats

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Abstract

Twelve male and female rats per group were exposed to the rubber accelerator 1,3-di-*o*-tolylguanidine (DTG) by gavage at 0, 8, 20 or 50 mg/kg bw/day. Males were dosed for a total of 49 days beginning 14 days before mating. Females were dosed for a total of 40–49 days beginning 14 days before mating to day 3 of lactation throughout the mating and gestation period. At 50 mg/kg bw/day, deaths were observed in two males and three females. Lowered body weight gain and food consumption were noted in males at 50 mg/kg bw/day and females at 20 and 50 mg/kg bw/day. Mydriasis, decreased locomotor activity, bradypnea, prone position, tremor and/or salivation were observed in males and females at 20 and 50 mg/kg bw/day. No effects of DTG were found on the estrous cyclicity, precoital interval, copulation, fertility and gestational indices, numbers of corpora lutea and implantations, or gestation length. A significant decrease in the number, body weight and viability of offspring and increase in the incidence of fetuses with external malformations were found at 50 mg/kg bw/day. Oligodactyly, anal atresia and tail anomalies were observed. These data suggest that DTG may be teratogenic. The NOAELs of DTG for general and developmental toxicity in rats are 8 and 20 mg/kg bw/day, respectively.

Keywords: Di-*o*-tolylguanidine; Rubber accelerator; Sigma ligand; Reproductive and developmental toxicity; Teratogenicity; Malformation; Rat

1. Introduction

The basic rubber accelerator 1,3-di-*o*-tolylguanidine (CAS No. 97-39-2; DTG) is produced in the million pound range annually in the United States [1,2]. DTG is known as a selective sigma ligand [3]. In this context, many pharmacological studies of DTG were performed [3–12]. Ligands that interact with sigma sites have been shown to produce hypothermia [4–6]. Hypothermia induced by DTG was detected following subcutaneous or intracerebroventricle injection in rats [5,6] and intraperitoneal injection in mice [4]. The intraperitoneal injection of DTG potently reduced the pain behavior in the acute but increased pain behavior in the tonic phase in the formalin test in mice [7]. Intraperitoneal injection of DTG produced significant but short-lived increases in the withdrawal latencies in mice [4]. Bastianetto et al. [8] showed that unilateral intranigral injection caused circulating behavior in rats and suggested that sigma sites play a role in movement and posture through their association with brainstem and forebrain motor control circuits. Decreased locomotor activity induced by intraperitoneal injection [9,10], increased bladder capacity induced by intravenous injection in the anaesthetized condition [11] and no change in immobility time in open field after intraperitoneal injection [12] were also reported in rats given DTG. Toxicological studies on DTG have given little information on acute animal toxicity [13]: intraperitoneal LD50 was 25 mg/kg bw in mice; oral LD50 was 500 mg/kg bw in rats; lowest published lethal dose of oral administration was 80 mg/kg bw in rabbits; and the lowest published lethal dose was 120 mg/kg bw after oral administration in mammals, species unspecified. At the present time, no information is available for the reproductive and developmental toxicity of DTG. It is generally assumed that the results of animal test on chemical toxicity are relevant to human health [14]. As such, the testing for reproductive and developmental toxicity

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in animal models is an important part of the overall toxicology. The present study was conducted to obtain information on the effects of DTG on reproductive and developmental parameters in rats.

2. Materials and methods

This study was performed in compliance with OECD guideline 421 Reproduction/Developmental Toxicity Screening Test [15] and in accordance with the principles for Good Laboratory Practice [16,17] and "Guidance for Animal Care and Use" of Panapharm Laboratories Co., Ltd.

2.1. Animals

International Genetic Standard (Crj: CD (SD) IGS) rats were used throughout this study. This strain was chosen because it is most commonly used in toxic studies, including reproductive and developmental toxicity studies, and historical control data are available. Males and females at 8 weeks of age were purchased from Atsugi Breeding Center, Charles River Japan, Inc. (Yokohama, Japan). The rats were acclimated to the laboratory for 13 days prior to the start of the experiment. Male and female rats found to be in good health were selected for use. Vaginal smears of each female were recorded and only females showing a 4-day estrous cycle were used in the experiment. Male and female rats were distributed on a random basis into four groups of 12 males and 12 females each. Rats were housed individually, except during the acclimation, mating and nursing periods. From day 0 of pregnancy to the day of sacrifice, individual dams and litters were reared using wooden chips as bedding (White Flake; Charles River Japan, Inc.).

Animals were reared on a sterilized basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and sterilized water ad libitum and maintained in an air-conditioned room at 24 ± 2 °C, with a relative humidity of 55 ± 10%, a 12-h light/12-h dark cycle and ventilation with 13–15 air charges per hour.

2.2. Chemicals and dosing

DTG was obtained from Sumitomo Chemical Co., Ltd. (Tokyo, Japan). DTG, a white powder, is slightly soluble in hot water and alcohol, soluble in chloroform and very soluble in ether, and its melting point is 179 °C, specific gravity is 1.10 and molecular weight is 239.3 [2]. The DTG (Lot No. 30J08) used in this study was 99.6% pure, and it was kept in a dark place at room temperature. The purity and stability of the chemical were verified by analysis before the study. Rats were dosed once daily by gastric intubation with DTG at a dose of 0 (control), 8, 20 or 50 mg/kg bw. The dosage levels were determined based on the results of our previous dose-finding study, the 14-day repeated dose toxicity study in rats given DTG by gavage at 0, 10, 20, 40 or 80 mg/kg bw/day, in which deaths were found at 80 mg/kg bw/day, decreased locomotor activity, mydriasis, tremor and salivation were observed at 40 and 80 mg/kg bw/day, and no adverse effects were detected at 10 and 20 mg/kg bw/day (data not shown). DTG was suspended in 0.5% (w/v) carboxymethylcellulose-Na solution with 0.1% (w/v) Tween 80. Males (12 rats/group) were dosed for a total of 49 days beginning 14 days before mating. Females (12 rats/group) were dosed for a total of 40–49 days beginning 14 days before mating to day 3 of lactation throughout the mating and gestation period. The volume of each dose was adjusted to 10 ml/kg body weight based on the latest body weight during the re-mating and mating period in males and females or the body weight on day 0 of pregnancy in females after copulation. Control rats were given 0.5% (w/v) carboxymethylcellulose-Na solution with 0.1% (w/v) Tween 80. The stability of formulations has been confirmed for up to 8 days. During use, the formulations were maintained under such conditions for less than 7 days, and the target concentration was 96.5 to 101.4%.

2.3. Observations

All rats were observed daily for clinical signs of toxicity. The body weight was recorded twice a week in males, and twice a week during the pre-mating and mating periods, on days 0, 7, 14 and 21 of pregnancy and on days 0 and 4 of lactation in females. Food consumption was recorded twice weekly during the pre-mating period in males, and twice weekly during the pre-mating period, on days 1, 7, 14 and 21 of pregnancy and on days 1 and 4 of lactation in females. The rats were euthanized by exsanguination under anesthesia on the next day of the last administration in males and on day 4 of lactation in females. The external surfaces of the rats were examined. The abdomen and thoracic cavity were opened, and gross internal examination was performed. In males, the testes and epididymides were weighed. In females, the numbers of corpora lutea and implantation sites and weight of the ovaries were recorded. The testes and epididymides were fixed with Bouin's solution and preserved in 10% neutral buffered formalin, and the ovaries were stored in 10% neutral buffered formalin. Histopathological evaluations were performed on hematoxylin–eosin-stained tissue sections of these organs.

Daily vaginal lavage samples of each female were evaluated for estrous cyclicity throughout the pre-mating period. Each female rat was mated overnight with a single male rat of the same dosage group until copulation occurred or the mating period, 2 weeks, had elapsed. During the mating period, daily vaginal smears were examined for the presence of sperm. The presence of the sperm in the vaginal smear and/or a vaginal plug was considered evidence for successful mating. Once insemination was confirmed, the females were checked for signs of parturition before noon from day 20 of pregnancy. The females were allowed to deliver spontaneously and nurse their pups until postnatal day (PND) 4. The day on which parturition was completed by 12:00 was designated as PND 0. Litter size and numbers of live and dead pups were recorded. Gender was determined on live pups examined grossly and individually weighed on PNDs 0 and 4. On PND 4, the pups were euthanized by exsanguination under anesthesia and gross internal examinations were performed.

2.4. Data analysis

The statistical analysis of pups was carried out using the litter as the experimental unit. The body weight, body weight gain, food consumption, length of estrous cycles, precoital interval, gestation length, weight of the organs, relative organ weight, numbers of corpora lutea, implantations and live and dead pups, total number of pups and weight of live pups were analyzed with Bartlett's test for homogeneity of variance at the 5% level of significance. If homogeneous the data were analyzed using Dunnett's multiple comparison test to compare the mean of the control group with that of each dosage group. If not, the DTG-treated groups were compared with that of the control group with Steel's multiple comparison test. The implantation, delivery and viability indexes, and incidence of pups with anomalies and individual anomalies were analyzed with Wilcoxon's rank sum test. The mortality, copulation, fertility and gestation indexes, and sex ratio of pups were analyzed with Fisher's exact test. The 5% level of probability was used as the criterion for significant.

3. Results

Table 1 shows the findings in male rats given DTG. At 50 mg/kg bw/day, one male died after six administrations and one male died after seven administrations. These dead rats showed mydriasis, decreased locomotor activity, bradypnea, a prone position and tremor 10–20 min after the administration of DTG. In surviving males, mydriasis, decreased locomotor activity, bradypnea and prone position on days 1–9 of the administration period, tremor during the whole period of administration and salivation on days 22–49 of the administration period were also observed at 50 mg/kg bw/day. Salivation was noted on days 28–49 of the administration period at 20 mg/kg bw/day. A significant decrease in the body weight gain was found on days 1–8 (81% decrease) and days 15–22 (48% decrease) of the administration period at 50 mg/kg bw/day. At this dose, significantly lower food consumption on days 7–8 (20% decrease) and days 14–15 (7% decrease) of the administration period was also observed.

Table 1
Findings in male rats given DTG

	Dose (mg/kg bw/day)			
	0 (control)	8	20	50
No. of male rats	12	12	12	12
No. of deaths during pre-mating period	0	0	0	2
Initial body weight (g)[a]	381 ± 16	379 ± 16	378 ± 15	380 ± 16
Body weight gain (g)[a]				
Days 1–8	30 ± 7	33 ± 7	25 ± 7	6 ± 9**
Days 8–15	29 ± 5	32 ± 5	32 ± 7	24 ± 7
Days 15–22	23 ± 6	25 ± 8	23 ± 7	12 ± 11**
Days 22–29	19 ± 9	22 ± 7	25 ± 8	19 ± 5
Days 29–36	22 ± 6	22 ± 6	23 ± 7	18 ± 8
Days 36–43	15 ± 8	12 ± 9	13 ± 5	14 ± 7
Days 43–50	19 ± 8	19 ± 7	13 ± 4	13 ± 11
Food consumption (g/day/rat)[a]				
Days 7–8	25 ± 3	26 ± 3	26 ± 2	20 ± 3**
Days 14–15	29 ± 2	30 ± 2	29 ± 3	27 ± 3*
Days 29–30	27 ± 2	27 ± 3	28 ± 3	25 ± 2
Days 35–36	28 ± 2	29 ± 2	29 ± 2	27 ± 2
Days 42–43	26 ± 3	25 ± 3	27 ± 4	27 ± 3
Days 49–50	28 ± 4	29 ± 3	28 ± 2	28 ± 3

[a] Values are given as the mean ± S.D.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).

Table 2 presents the findings in female rats given DTG. At 50 mg/kg bw/day, two females died after the first administration and one female died after normal delivery of her pups on day 22 of pregnancy. Mydriasis, decreased locomotor activity, bradypnea, prone position, and tremor and salivation 10–20 min after the administration of DTG were observed in females died after the first administration. These clinical signs and salivation were found during pregnancy and on day of parturition in a female which died after parturition. In surviving females, mydriasis, decreased locomotor activity, bradypnea and prone position on day 1 of the administration period to day 0 of lactation, tremor on day 1 of the administration period to day 5 of pregnancy and salivation on day 4 of pregnancy to day 3 of lactation were observed at 50 mg/kg bw/day. Mydriasis, decreased locomotor

Table 2
Findings in female rats given DTG

	Dose (mg/kg bw/day)			
	0 (control)	8	20	50
No. of female rats	12	12	12	12
No. of deaths during pre-mating period	0	0	0	2
No. of deaths during pregnancy	0	0	0	1
Initial body weight (g)[a]	381 ± 16	379 ± 16	378 ± 15	380 ± 16
Body weight gain (g)[a]				
Days 1–8	19 ± 8	17 ± 7	11 ± 6*	−1 ± 9**
Days 8–15	10 ± 7	15 ± 8	20 ± 5**	15 ± 10
Days 0–7 of pregnancy	34 ± 6	31 ± 6	33 ± 4	28 ± 8
Days 7–14 of pregnancy	34 ± 5	34 ± 4	36 ± 3	30 ± 10
Days 14–21 of pregnancy	85 ± 17	100 ± 14	105 ± 9*	42 ± 21**
Days 0–4 of lactation	20 ± 19	14 ± 16	22 ± 9	16 ± 13
Food consumption (g/day/rat)[a]				
Days 7–8	22 ± 3	21 ± 2	19 ± 2**	13 ± 3**
Days 14–15	20 ± 4	22 ± 3	22 ± 2	20 ± 2
Days 6–7 of pregnancy	22 ± 3	23 ± 2	23 ± 3	17 ± 3**
Days 13–14 of pregnancy	23 ± 2	24 ± 3	25 ± 2	22 ± 5
Days 20–21 of pregnancy	24 ± 4	26 ± 3	29 ± 3*	21 ± 5
Days 3–4 of lactation	41 ± 5	41 ± 3	46 ± 4*	32 ± 6**

[a] Values are given as the mean ± S.D.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).

Table 3
Reproductive findings in rats given DTG

	Dose (mg/kg bw/day)			
	0 (control)	8	20	50
No. of pairs	12	12	12	10
Length of estrous cycles (day)[a]	4.0 ± 0.2	4.1 ± 0.3	4.1 ± 0.3	4.1 ± 0.2
Precoital interval (day)[a]	3.0 ± 1.0	2.7 ± 1.0	2.4 ± 1.1	2.2 ± 1.0
Copulation index (%)[b]				
Male	100	91.7	100	100
Female	100	91.7	100	100
Fertility index (%)[c]	100	100	91.7	100
Gestation index (%)[d]	100	100	100	90.0
Gestation length (day)[a]	22.6 ± 0.5	22.3 ± 0.5	22.5 ± 0.5	22.6 ± 0.5
Weight of testes (g)[a]	3.24 ± 0.34	3.34 ± 0.19	3.31 ± 0.28	3.30 ± 0.24
Relative weight of testes[a,e]	0.60 ± 0.05	0.62 ± 0.07	0.63 ± 0.07	0.68 ± 0.07*
Weight of epididymides (g)[a]	1.16 ± 0.10	1.21 ± 0.06	1.21 ± 0.12	1.23 ± 0.07
Relative weight of epididymides[a,e]	0.22 ± 0.02	0.22 ± 0.02	0.23 ± 0.03	0.25 ± 0.02**
Weight of ovaries (mg)[a]	101 ± 8	106 ± 6	101 ± 11	102 ± 10
Relative weight of ovaries[a,e]	30 ± 2	31 ± 2	28 ± 3	32 ± 2

[a] Values are given as the mean ± S.D.
[b] Copulation index (%) = (no. of rats copulated/no. of pairs) × 100.
[c] Fertility index (%) = (no. of females pregnant/no. of females copulated) × 100.
[d] Gestation index (%) = (no. of females with parturition/no. of females copulated) × 100.
[e] Relative weight = organ weight/100 g of body weight.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).

activity, bradypnea and prone position on days 2–3 of the administration period, and salivation on day 14 of pregnancy to day 3 of lactation were observed at 20 mg/kg bw/day. Body weight gain was significantly lowered on days 1–8 of the pre-mating period at 20 mg/kg bw/day (42% decrease) and on days 1–8 of the pre-mating period (105% decrease) and days 14–21 of pregnancy (49% decrease) at 50 mg/kg bw/day. At 20 mg/kg bw/day, a significantly higher body weight gain was observed on days 8–15 of the pre-mating period and days 14–21 of pregnancy. Food consumption was significantly reduced on days 7–8 of the pre-mating period at 20 mg/kg bw/day (14% decrease) and on days 7–8 of the pre-mating period (41% decrease) and days 3–4 of lactation (24% decrease) at 50 mg/kg bw/day. At 20 mg/kg bw/day, a significant increase in the food consumption was observed on days 20–21 of pregnancy and days 3–4 of lactation.

The reproductive findings in rats given DTG are presented in Table 3. No effects of DTG were observed on the length of estrous cycles, precoital interval and gestation length. One pair did not copulate at 8 mg/kg bw/day, one female did not become impregnated at 20 mg/kg bw/day and one female did not deliver any pups at 50 mg/kg bw/day; however, no significant differences were noted in the copulation, fertility or gestation index between the control and DTG-treated groups. The weights of the testes and epididymides, and absolute weight and relative weight of the ovaries in the DTG-treated groups did not differ from the control group. The relative weights of the testes (13% increase) and epididymides (14% increase) were significantly higher at 50 mg/kg bw/day.

The developmental findings in rats given DTG are shown in Table 4. There was no significant difference in the numbers of corpora lutea, implantations and stillborns, implantation index, sex ratio of live pups, viability index on day 0 of lactation and body weight of live pups on day 4 of lactation between the control and DTG-treated groups. The numbers of pups delivered (45% decrease) and live pups delivered (45% decrease) and delivery index (43% decrease) were significantly lowered at 50 mg/kg bw/day. At this dose, the viability index on day 4 of lactation (34% decrease) and body weight of live male (16% decrease) and female (19% decrease) pups on day 0 of lactation were also significantly decreased. Two dams with totally litter loss were observed. No poor maternal behavior or nursing was observed in dams at 50 mg/kg bw/day. No histopathological changes were found in the testes, epididymides and ovaries in the DTG-treated groups. External anomalies in pups of rats given DTG are also presented in Table 4. No fetuses with external malformations were observed in the control and groups given DTG at 8 and 20 mg/kg bw/day. At 50 mg/kg bw/day, fetuses with external malformations were found in 10 out of the 65 fetuses and in 3 out of the 9 litters. Oligodactyly was observed in four pups in two litters. A kinked tail was found in six pups in one litter and a short tail and anal atresia was observed in one pup in each letter. Although there was no significant difference in the incidence of fetuses with individual malformations between the control and 50 mg/kg bw/day groups, a significantly higher incidence of total number of fetuses with external malformations was noted at this dose.

Table 4
Developmental findings in rats given DTG

	Dose (mg/kg bw/day)			
	0 (control)	8	20	50
No. of litters	12	11	11	9
No. of implantations[a]	14.3 ± 2.6	16.2 ± 1.9	15.9 ± 1.4	14.2 ± 3.6
Implantation index (%)[b]	92.2	94.7	97.6	90.9
No. of pups delivered[a]	13.0 ± 2.4	15.2 ± 2.0	14.7 ± 1.4	7.2 ± 4.1**
No. of live pups delivered[a]	13.0 ± 2.4	15.1 ± 1.9	14.7 ± 1.4	7.2 ± 4.1**
No. of stillborns	0	0.1 ± 0.3	0	0
Delivery index (%)[c]	91.0	93.3	92.2	51.7**
Sex ratio of live pups (males/females)	71/85	84/82	80/82	31/34
Viability index (%)[d,e]				
Day 0 of lactation	100	99.5	100	100
Day 4 of lactation	99.4	99.4	100	65.4**
Body weight of male pups during lactation (g)[a]				
Day 0	7.4 ± 0.7	6.9 ± 0.6	7.3 ± 0.6	6.2 ± 1.0**
Day 4	11.9 ± 1.3	11.1 ± 1.0	11.7 ± 1.0	11.0 ± 2.3
Body weight of female pups during lactation (g)[a]				
Day 0	7.0 ± 0.7	6.6 ± 0.6	6.8 ± 0.7	5.7 ± 0.8**
Day 4	11.4 ± 1.3	10.5 ± 1.0	11.0 ± 0.9	10.5 ± 2.0
External examination of pups				
No. of pups (litters) with malformations	0	0	0	10 (3)*
Oligodactyly	0	0	0	4 (2)
Kinky tail	0	0	0	6 (1)
Short tail	0	0	0	1
Anal atresia	0	0	0	1

[a] Values are given as the mean ± S.D.
[b] Implantation index (%) = (no. of implantations/no. of corpora lutea) × 100.
[c] Delivery index (%) = (no. of live pups delivered/no. of implantations) × 100.
[d] Viability index on day 0 of lactation (%) = (no. of live pups delivered/total no. of pups delivered) × 100.
[e] Viability index on day 4 of lactation (%) = (no. of live pups on day 4 of lactation/no. of live pups delivered) × 100.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).

4. Discussion

The present study was conducted to obtain initial information on the possible effects of DTG on reproduction and development in rats. The data show that DTG exerts developmental toxicity and suggest that DTG possesses teratogenic potential.

DTG was given to males during the pre-mating and mating periods and to females during the pre-mating, mating, pregnancy and shortly after parturition. The dosage used in the present study was sufficiently high such that it should be expected to induce general toxic and neurobehavioral effects. As expected, general toxicity, such as decreases in body weight gain and food consumption, was found at 50 mg/kg bw/day in males and at 20 and 50 mg/kg bw/day in females. Decreases in the body weight gain and food consumption during the early administration period, and thereafter, significant increases in body weight gain and food consumption were observed in females at 20 mg/kg bw/day. One possible explanation for increased body weight gain during late pregnancy at 20 mg/kg bw/day may be higher number of pups and higher net weight gain during pregnancy at this dose compared with the controls. Such recovery did not occur at the highest dose. Neurobehavioral effects, such as mydriasis, decreased locomotor activity, bradypnea, prone position, tremor and salivation, were also observed at 20 and 50 mg/kg bw/day. DTG is a specific sigma receptor ligand [3] and sigma receptor ligands can modulate neurotransmissions, including the noradrenergic, glutamatergic and dopaminergic system [10,18,19]. It was reported that systemic injection of DTG caused neurobehavioral changes in rats [5,6,9,10]. The present study shows that the oral administration of DTG also induces neurobehavioral changes, and it is neurobehaviorally toxic at 20 and 50 mg/kg bw/day in rats.

Higher relative weights, but not the absolute weight, of the testes and epididymides were observed at 50 mg/kg bw/day. Body weights of male rats on the day of scheduled sacrifice were 537 and 485 g in the control and 50 mg/kg bw/day groups, respectively. It seems likely that the higher relative weights of the testes and epididymides at the highest dose were due to secondarily lowered body weight but not due to the direct effects of DTG on the male reproductive organs. Other male reproductive parameters were not significantly changed, even at the highest dose. These findings suggest that DTG is not reproductively toxic to male rats. It seems unlikely that DTG exerts reproductive toxicity to female rats when administered during the pre-mating, mating, pregnancy and early lactation period, because no adverse effects on the maternal reproductive parameters, including estrous cyclicity, precoital interval, copulation

index, fertility index, gestation index, gestation length and ovarian weight, were caused by the administration of DTG in females.

As for the developmental indexes, decreases in the numbers of total pups and live pups delivered, delivery index, viability on PND 4 and body weight of live pups on PND 0 were detected at 50 mg/kg bw/day. These findings indicate that DTG is toxic to the survival and growth of offspring and exerts developmental toxicity at 50 mg/kg bw/day in rats.

In the present study, the teratogenic effect of DTG is strongly suggested by the external examinations of pups. At 50 mg/kg bw/day, a significant increase in the total number of fetuses with external malformations was noted; however, incidences of fetuses with individual types of external malformations at this dose were not significantly different from those in the control group. The external malformations observed in the present study are of the types that occur spontaneously among control rat fetuses reported in the literature [20–23]. In the present study, only external examination in the newborn rats was performed, and no internal or skeletal examinations were performed. Even animals not ordinarily carnivorous, including nonhuman primates, are likely to eat dead and moribund offspring, as well as those with malformations that involve skin lesions allowing the loss of body fluids or the exposure of viscera [24]. To accurately evaluate the prenatal developmental toxicity including teratogenicity, it is necessary to interrupt pregnancy 12–24 h before the expected term either by hysterectomy or the necropsy of maternal animals [24,25]. The present study was performed in compliance with OECD guideline 421 Reproduction/Developmental Toxicity Screening Test [15], and this screening test guideline does not provide complete information on all aspects of reproduction and development due to the relatively small numbers of animals in the dose groups and selectivity of the endpoints. In order to further evaluate the developmental toxicity, including teratogenicity, of DTG in rats, a prenatal developmental toxicity study is currently in progress.

In conclusion, DTG caused decreased body weight gain and food consumption at 50 mg/kg bw/day in males and at 20 and 50 mg/kg bw/day in females, neurobehavioral changes at 20 and 50 mg/kg bw/day in both sexes, and changes in developmental parameters at 50 mg/kg bw/day. DTG is suggested to be teratogenic. The NOAELs of DTG for general and developmental toxicity were 8 and 20 mg/kg bw/day, respectively, in rats.

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SEMI-QUANTITATIVE IMMUNOHISTOCHEMICAL ANALYSIS OF MALE RAT-SPECIFIC α_{2u}-GLOBULIN ACCUMULATION FOR CHEMICAL TOXICITY EVALUATION

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ABSTRACT — We purified male rat urinary α_{2u}-globulin, prepared the antibody in rabbits, and improved an immunohistochemical detection method using this antibody for male rat-specific α_{2u}-globulin accumulation appearing as hyaline droplets in the kidneys. Our prepared antibody reacted specifically with α_{2u}-globulin in both immunohistochemical and Western blotting analyses, furthermore, and the graded immuno-reactivities on the slide were well associated with computational image analyzing results. Using this method, we retrospectively analyzed the renal sections from the toxicity studies of 12 nephrotoxic chemicals, which had already been conducted under the Japanese Existing Chemicals Survey Program. We demonstrated that the hyaline droplets induced by treatment with 10 chemicals (1,4-dibromobenzene, dicyclopentadiene, 3,4-dimethylaniline, 1,4-dicyanobenzene, tetrahydrothiophene-1,1-dioxide, 1,3-dicyanobenzene, acenaphthene, 3,4-dichloro-1-butene, 3a,4,7,7a-tetrahydro-1H-indene and 3,5,5-trimethylhexan-1-ol) were directly associated with α_{2u}-globulin accumulation. This immunohistochemical method is convenient for applying, even retrospectively, paraffin sections from general toxicity studies and could be useful for qualifying male rat-specific hyaline droplets consisting of α_{2u}-globulin and renal risk in humans.

KEY WORDS: α_{2u}-globulin, Immunohistochemistry, Hyaline droplet, Nephrotoxicity

INTRODUCTION

For risk assessment of chemicals, the most critical data are derived from animal toxicity studies because of a general lack of information on humans. Although all available results from animal studies have been applied to human risk assessment, in principle, exclusion of some specific toxicities, which might not occur in humans, should be taken into account. Among laboratory animals, the rat has been commonly used for toxicity studies, especially sub-acute, long-term or carcinogenicity studies. Nephropathy with hyaline droplets and renal tubular neoplasia caused by chemicals inducing α_{2u}-globulin accumulation (CIGA) are considered to be a male rat-specific toxicity, not occurring in female rats or other animals, including primates. Although low molecular proteins homologous to α_{2u}-globulin can be detected in other species, including mice and humans, none of these proteins have been confirmed to bind to CIGA, followed by accumulation of the protein-CIGA complex as in the case of α_{2u}-globulin. It is therefore believed that renal toxicity induced by CIGA in male rats is unlikely to occur in humans (Hard *et al.*, 1993).

α_{2u}-Globulin was first identified in male rat urine (Roy and Neuhaus, 1966), and had been reported to be a male rat-specific protein with a molecular weight of 18 to 20 kDa. The major source of urinary α_{2u}-globulin

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is the liver, where α_{2u}-globulin mRNA constitutes approximately 1% of the total hepatic mRNA (Sippel *et al.*, 1976; Kurtz and Feigelson, 1977). Neither α_{2u}-globulin nor its mRNA is detectable in the female liver (Sippel *et al.*, 1975, 1976; MacInnes *et al.*, 1986). The blood α_{2u}-globulin secreted from the liver is freely filtered through the glomerulus, and in mature rats, about two-thirds of the filtered protein is reabsorbed by tubules and the remainder is excreted through the urine (Neuhaus *et al.*, 1981). CIGA binds noncovalently to α_{2u}-globulin, and the resulting complex shows less degradability with proteolytic enzymes, causing an accumulation of the complex that is detectable as hyaline droplets with a light microscope. Various chemicals have been suspected of being CIGA based on detection of the evidence for exacerbation of hyaline droplets in renal proximal tubules in male rats, though not in females. Direct evidence for increasing α_{2u}-globulin levels has been demonstrated for only a few of these chemicals, however, including 2,2,4-trimethylpentane (Stonard *et al.*, 1986; Charbonneau *et al.*, 1987; Lock *et al.*, 1987), decalin (Kanerva *et al.*, 1987), d-limonene (Lehman-McKeeman *et al.*, 1989; Webb *et al.*, 1989), 1,4-dichlorobenzene (Charbonneau *et al.*, 1989), isophorone (Strasser *et al.*, 1988), lindane (Dietrich and Swenberg, 1990), tri- or per-chloroethylene and pentachoroethane (Goldsworthy *et al.*, 1888).

A number of initial safety assessments has so far been conducted for industrial chemicals, including both new and existing chemicals by the Japanese government or the OECD high production volume chemicals programs. Certain chemicals among these industrial chemicals have been suspected of being CIGA. In some cases, however, renal changes in male rats have been assessed as the endpoint for extrapolation to human health risk owing to a lack of direct evidence caused by α_{2u}-globulin accumulation, because no antibody against α_{2u}-globulin is commercially available for general toxicity studies. Some immunohistochemical α_{2u}-globulin analysis methods had already been developed (Burnett *et al.*, 1989; Hashimoto and Takaya, 1992; Caldwell *et al.*, 1999). As these methods required glycolmethacrylate embedding or specific computational analysis, they would be inappropriate for confirming α_{2u}-globulin accumulation in routinely conducted guideline-based toxicity studies. We therefore improved an immunohistochemical α_{2u}-globulin detection system using paraffin sections, which are generally used for standard toxicity studies. We evaluated the several chemicals suspected of being CIGA, moreover, and indicated the direct evidence caused by α_{2u}-globulin accumulation.

MATERIALS AND METHODS

Preparation of anti α_{2u}-globulin antibody

α_{2u}-globulin as an antigen was obtained from the urine collected from aged male rats, pooled, and used to immunize rabbits. The immunization procedures, including the amount of antigen and immunizing intervals, were determined from the results of a preliminary test referring to the methods of Kurtz *et al.* (1976). The antigen was injected under the skin at a dose of 1 mg/animal (1st injection) or 0.5 mg/animal (2nd and subsequent injections) once at two weeks. Blood sampling was conducted periodically and the antibody titer measured. When the antibody titer level reached a plateau, whole blood was collected and antiserum was obtained from the blood. The antiserum was used for immunohistochemistry and immuno-electron microscopy. For measurement of the α_{2u}-globulin content in the urine and tissues, the antibody was purified from the antiserum using a DEAE ionic exchange column after ammonium sulfate precipitation. The singularity of the antibody was confirmed as a single diffuse band of approximately 19 kDa by Western blotting analysis. This study and the following study were carried out in accordance with the Law for the Humane Treatment and Management of Animals and the Standards Relating to the Care and Management, etc. of Experimental Animals in Japan.

Experiment 1 Confirmation of specific reactivity of the antibody to α_{2u}-globulin

1. Preparation of α_{2u}-globulin nephropathy rats

To confirm the specific reactivity of the anti-α_{2u}-globulin antibody, we prepared α_{2u}-globulin nephropathy rats as follows. Male and female Crj:CD(SD)IGS rats were obtained from Charles River Japan Inc. and used at the age of 11 weeks. d-Limonene (Nacalai Tesque Inc.), a well-known α_{2u}-globulin nephropathy inducer, was administered to the rats, consisting of 4 males and 4 females each, for 10 days at doses of 0, 150 and 300 mg/kg/day by gavage using corn oil as a vehicle. The rats were housed individually in stainless steel wire cages in an animal room with a controlled temperature of 24±2°C, humidity of 55±10% and a 12-hr light/dark cycle (lighting from 7:00 to 19:00) and allowed access to food and water ad libitum.

Pooled urine was collected for 24 hr on the day before the start of administration and on Day 9 of administration. After the 10-day administration period,

the rats were anesthetized with intraperitoneal injection of 30 mg/kg of sodium pentobarbital and perfused with physiological saline-added lactose (Lactec, Otsuka Pharmaceutical Factory Inc.) through the sinus aortae, after which the liver and kidneys were removed. The urine and a part of the liver and kidneys were used for measurement of their α_{2u}-globulin content and the remainder of the liver and kidneys for histopathology, immunohistochemistry and immuno-electron microscopy. The samples for histopathology and immunohistochemistry were embedded in paraffin following fixation with 10% neutral buffered formalin solution for about two weeks. The samples for immuno-electron microscopy were dehydrated with an ascending series of ethanol and embedded in spurr resin following pre- and post-fixation with 2.5% glutaraldehide and 1% osmium tetroxide solutions, respectively.

2. Histopathology and immunohistochemistry

The serial paraffin sections were prepared, deparaffinized and then stained with hematoxylin and eosin (HE) accompanied by Azan-Mallory staining and periodic acid shiff(PAS) reaction.

For immunohistochemistry, the paraffin sections were deparaffinized and incubated with 0.25% pronase E for 20 min at 37°C, after which they were washed 3 times in Tween-PBS (PBS containing 0.1% Tween 20, pH7.6). The specimens were incubated with 0.3% H_2O_2 in methanol at room temperature for 30 min to inactivate the endogenous peroxidase activity, and then washed 3 times in Tween-PBS. After blocking against nonspecific immuno-reactions with 10% FCA was conducted at room temperature for 20 min, the sections were incubated overnight with rabbit anti-α_{2u}-globulin antiserum at 4°C at a dilution of 1:80000 in PBS containing 1% BSA. Negative controls were incubated with an equivalent volume of diluent solution alone. The sections were washed 3 times in Tween-PBS and incubated with biotynilated secondary antibody (goat anti-rabbit and goat anti-mouse immunoglobulins, Dako, LSAB2 kit) at room temperature for 30 min. After they were washed 3 times inTween-PBS, the sections were incubated with horseradish peroxidase (HRP)-labelled streptavidin (Dako, LSAB2 kit) at room temperature for 30 min. The sections were then washed 3 times in PBS and reacted with 3,3-diaminobenzidine (DAB) for 5 min. The reactions were quenched by placement in running tap water, and the sections were then counterstained lightly with methylgreen, dehydrated in n-butanol, cleaned in xylene, and mounted.

3. Immuno-electron microscopy

Ultra-thin sections were prepared and reacted overnight with the anti-α_{2u}-globulin antiserum at a dilution of 1:5000 at 4°C. Protein A-colloidal Gold (10 nm, British Bio Cell International Inc.) was used at a dilution of 1:10, after which the sections were double stained with uranyl acetate and lead citrate.

4. Measurement of α_{2u}-globulin content in the liver, kidneys and urine

The α_{2u}-globulin content was measured in the liver and kidneys in all males in all the groups of α_{2u}-globulin nephropathy rats, and in the urine in two males each in the control and highest dose groups. The liver and kidneys were homogenized with phosphate buffer weighing 4 times their tissue weights and centrifuged at 105,000 g for one hour. The protein content of the supernatant thus obtained was measured for every molecular weight and the urine was measured similarly as is. Western blotting was then conducted using purified anti-α_{2u}-globulin antibody and the content of the protein showing a positive reaction was regarded as α_{2u}-globulin content.

Experiment 2 α_{2u}-globulin analysis for industrial chemicals

The selected chemicals are listed in Table 1. We selected 10 chemicals, which are suspected of being CIGA, among all the chemicals in the Japanese Existing Chemicals Survey Program (JECSP). In addition, two chemicals which caused renal toxicity without hyaline droplet accumulation were selected as negative controls. We used paraffin-embedded renal specimens originating from the JECSP toxicity studies conducted in several laboratories and stored for four to seven years in each. For each toxicity study, three groups (the control and low- and high-dose groups for 11 chemicals) or two groups (the control and high-dose groups for the other) were selected. The low-dose group has the dose showing the lowest effect for hyaline droplets in tubules or other renal changes, and the high-dose group has the highest dose administered in each toxicity study. The doses selected for each chemical are described in Table 1. Three male specimens were arbitrarily selected for each dose group based on the results obtained from HE-stained sections in the original studies.

The serial paraffin sections were prepared, deparaffinized and then stained with HE accompanied by Azan-Mallory staining and PAS reaction. The sections were also stained immunohistochemically using anti-

Table 1. Chemical name and effect dose derived from the general toxicity studies.

Chemical	Test type	Original study doses (mg/kg/day)	Effect doses (mg/kg/day) [a]			Original reported NOEL (mg/kg/day) [a]	The selected doses for analyzing (contr./low/high) (mg/kg/day)
			Histopathological findings		Non histopathological observations		
			AN	Other			
1,4-Dibromobenzene	RD	0/ 4/ 20/100/500	20≤ / –	100≤	100≤ / 20≤	4	0/ 20/500
Dicyclopentadiene	RT	0/ 4/ 20/100	4≤ / –	20≤ / 100	20≤ / 100	<4 / 20	0/ 4/100
3,4-Dimethylaniline	RD	0/10/ 50/250	50≤ / –	250	250 / 50≤	10	0/ 50/250
1,4-Dicyanobenzene	RD	0/ 1.25/ 5/ 20/ 80	5≤ / –	20≤ / –	20≤	1.25 / 5	0/ 5/ 80
Tetrahydrothiophene-1,1-dioxide	RD	0/60/ 200/700	200≤ / –	–	700	60 / 200	0/200/700
1,3-Dicyanobenzene	RD	0/ 8/ 40/200	8≤ / –	40≤ / 200	40≤	<8 / 8	0/ 8/200
Acenaphthene	RD	0/12/ 60/300	60≤ / –	300	300 / 60≤	12	0/ 60/300
3,4-Dichloro-1-butene	RT	0/ 0.4/ 2/ 10/ 50	10≤ / –	50	10≤ / 50	2 / 10	0/ 10/ 50
3a,4,7,7a-Tetrahydro-1*H*-indene	RT	0/ 67/200/600	67≤ / –	600	67≤ / 200≤	<67 / 67	0/ 67/600
3,5,5-Trimethylhexan-1-ol	RT	0/ 12/ 60/300	12≤ / –	60≤	60≤	12	0/ 12/300
2,4-di-*tert*-butylphenol	RD	0/ 5/ 20/ 75/300	– / –	300	300 / 75≤	75 / 20	0/ – /300
4-aminophenol	RD	0/ 4/ 20/100/500	– / –	100≤	100≤	20	0/100/500

[a] The data were described in a pattern of male/female when the data were different between the male and female.
RD, 28-day Repeat Dose Toxicity Test; RT, Combined Repeat Dose and Reproductive/Developmental Toxicity Test.
AN, α_{2u}-globulin nephropathy including hyaline droplets and subsequent tubular alteration.

α_{2u}-globulin antiserum by the above-mentioned protocol. HE-stained sections were used to examine the degree of hyaline droplets and to determine whether or not other findings were present. The degree of occurrence of hyaline droplets was divided into five grades, including none (−), minimal (±, barely detectable minimal appearance), slight (+, multifocal but not dispersed appearance), moderate (++, dispersed appearance over the cortex) and severe (+++, diffused appearance over the whole cortex). The staining sections with PAS, Azan-Mallory and anti-α_{2u}-globulin reaction were also graded similarly for positive-stained droplets. In addition, computational image analysis was carried out to verify the above-mentioned grading criteria using three typical immuno-stained samples for each grade. Images including almost all the renal superficial cortex were captured using a light microscope (Olympus BHS) and a digital camera (Olympus DP12). The captured images were measured for positive area using an image analyzing system (C-Imaging System, Compix Inc.), and the positive area (%) was then calculated from the data.

RESULTS

Experiment 1 Specific reactivity of the antibody to α_{2u}-globulin

On the HE-stained sections of the kidneys, hyaline droplets with round to irregular shapes were observed in the renal proximal tubular epithelium only in males administered d-limonene (Photo. 1a). The hyaline droplets were negative for PAS reaction (Photo 1b) but stained positively with Azan-Mallory staining (Photo 1c). With immuno-staining with the anti-α_{2u}-globulin antibody, the hyaline droplets were more clearly stained and more distinguishable than with Azan-Mallory staining (Photo 1d). The hyaline droplets showed a dose-dependent increase on the HE-stained sections (Photo 2, a-c) and positive reactions for hyaline droplets showed a correlational increase with immuno-staining (Photo 2, d-f). Very fine positive granules were also detected on the immuno-stained sections for all the males as background, but no positive reactions were observed in other tissue components. This background was observed generally in male kidneys and was, therefore, excluded from the grading in experiment 2. In the liver, all the males showed a positive reaction for the antibody in centrilobular hepatocytes. The degree of intensity was weaker than in the kidneys, and there was no clear intensification by d-limonene. No positive reaction for the anti-α_{2u}-globulin antibody was detected in the liver or kidneys in any females.

With electron microscopy, electron-dense and irregular-shaped inclusions surrounded by a single membrane were observed as changes corresponding to the hyaline droplets in the renal proximal tubular epithelium, and positive reactions were observed for the antibody with post-embedding method in the inclusions (Photo 3). A similar positive reaction was observed in the lysosomes of the renal tubule epithelium, but no positive reaction was detected in the hepatocytes.

The α_{2u}-globulin content in the kidneys of the males was increased dose-dependently by administration with d-limonene (Fig. 1). A dose-dependent but mild increase in α_{2u}-globulin content was also observed in the liver of the males. While no dose-dependent increase in the urine was noticeable, a lower molecular type of α_{2u}-globulin appeared in the males in the highest dose group, with the α_{2u}-globulin type reported as an early marker for α_{2u}-globulin nephropathy (Saito *et al.* 1991).

Experiment 2 α_{2u}-globulin analysis for industrial chemicals

Table 2 indicates the grades of all the samples with respect to hyaline droplets, positive droplets and immunological positive droplets analyzed with HE, Azan-Mallory and anti-α_{2u}-globulin antibody staining, respectively. In the controls there was a minimal to moderate amount of hyaline droplets in some animals and consequent variation for Azan-Mallory and anti-α_{2u} -globulin reaction. This variation was due to the arbitrary sampling of specimens, or probably related to the lot of the animals or to the difference of food used in each study. Dose-dependent increases of hyaline droplets in the renal proximal tubular epithelium were, however, confirmed for HE-staining of 10 chemicals suspected of being CIGA (1,4-dibromobenzene, dicyclopentadiene, 3,4-dimethylaniline, 1,4-dicyanobenzene, tetrahydrothiophene-1,1-dioxide, 1,3-dicyanobenzene, acenaphthene, 3,4-dichloro-1-butene, 3a,4,7,7a-tetrahydro-1H-indene, 3,5,5-trimethylhexan-1-ol). This was described in the original reports (Toxicity Testing Reports of Industrial Chemicals), although the occurrence of hyaline droplets varied in shape, size and number/cell with chemicals and showed no clear common features. In the highest dose groups of these chemicals, basophilic tubules, granular casts in the tubules and/or tubular dilatation were intensified or occurred as in the original reports. These changes

showed similar features in spite of the various severity and incidence with the chemicals. In serial sections prepared simultaneously, Azan-Mallory-positive reactions for hyaline droplets were detected dose-dependently in these 10 chemicals. No PAS-positive reaction was detected in any chemical. These staining behaviors of the hyaline droplets were the same as those in the case of d-limonen described above. Immunohistochemical staining using the anti-α_{2u}-globulin antibody revealed thoroughly dose-dependent positive reactions for hyaline droplets in all these chemicals. The resulting grades from three types of analysis were the same, demonstrating that a highly positive correlation exists among the three staining methods. As for the remainder not suspected of being CIGA (2,4-di-tert-butylphenol, 4-aminophenol), there was no increase of hyaline droplets or positive immunohistochemical reactions in any dose groups, as well as no stain in either PAS or Azan-Mallory staining. In addition, computational image analysis using three typical immuno-stained sections for each grade (Photo 4) showed a close correlation between the quantitative analysis and semi-quantitative grading (Fig. 2).

DISCUSSION

Many toxicity studies using laboratory animals have been conducted on environmental and industrial chemicals to ensure their safety or toxicity levels concerning human health. On extrapolating the results to humans, toxic mechanisms that are unlikely to occur in humans should be taken into account. A typical example of such toxicities is α_{2u}-globulin-related nephropathy and the consequent renal tumorigenesis in repeated

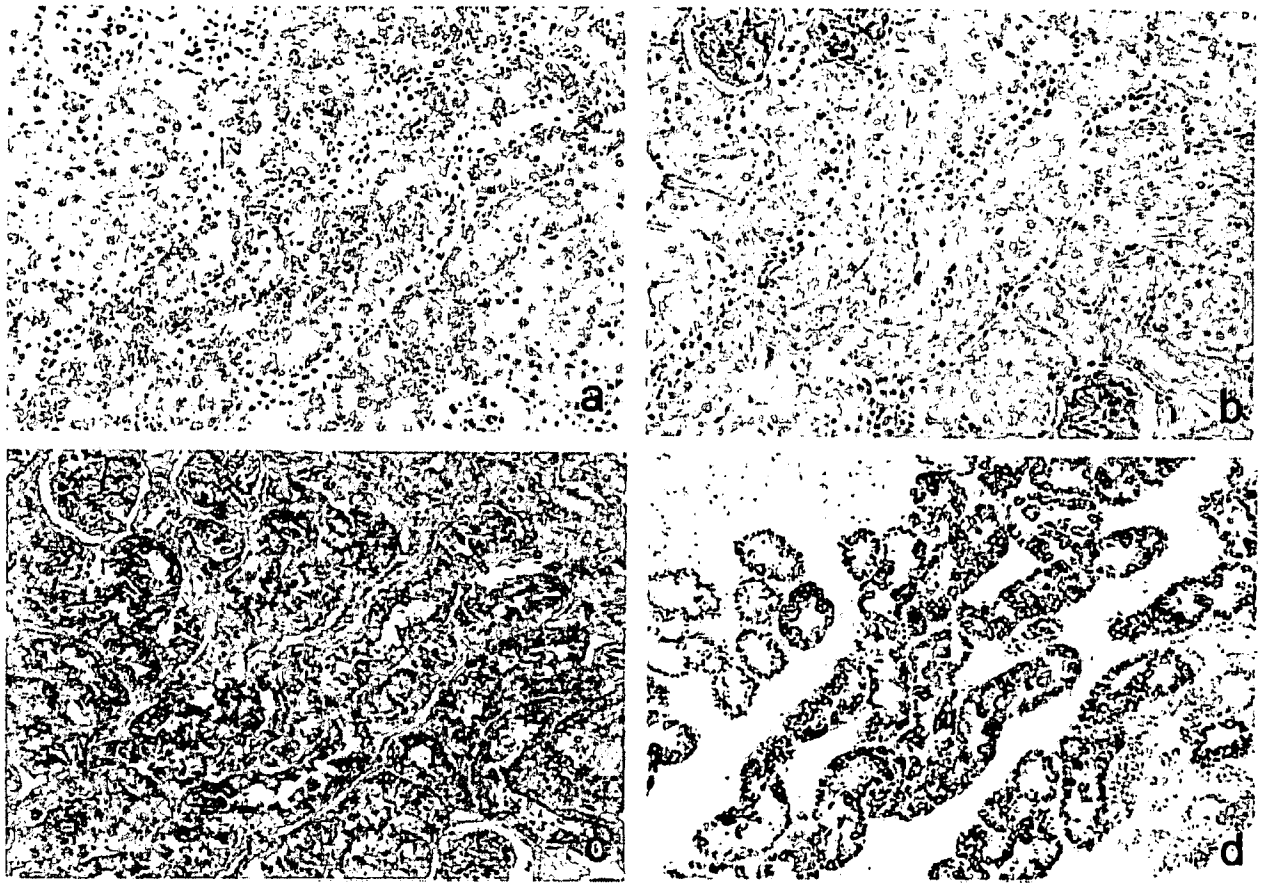

Photo 1. d-Limonene induced hyaline droplet accumulation in the kidney (HE, a). The hyaline droplets were PAS-negative(b), but they were stained positively with Azan-Mallory staining (c). Immunohistochemistry using the anti-α_{2u}-globulin antibody showed a clear positive reaction consistent with the hyaline droplets (d). Original magnification, ×66.

dose toxicity studies using male rats. This male rat-specific nephrotoxicity is not considered to occur in humans (Hard *et al.*, 1993). To exclude this male rat-specific toxicity from chemical risk assessment, it is necessary to demonstrate properly that such renal toxicity results from α_{2u}-globulin-CIGA complex accumulation. Detection analysis of α_{2u}-globulin in the nephrotoxicity has not been conducted in most conventional toxicity studies, however, especially in sub-acute toxicity screening studies for industrial chemicals. As

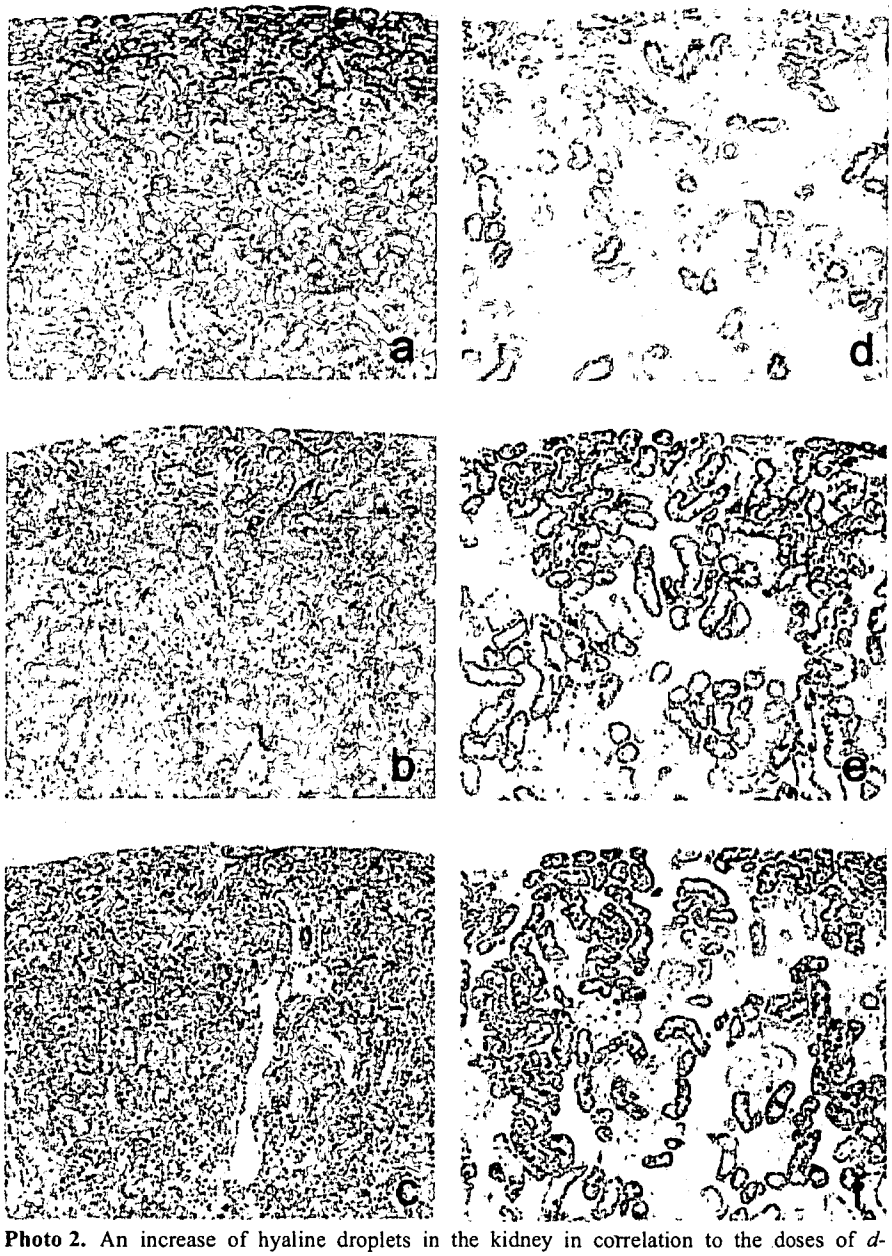

Photo 2. An increase of hyaline droplets in the kidney in correlation to the doses of *d*-limonene(HE, a - c). Positive reaction for the anti-α_{2u}-globulin antibody also increased with similar dose dependency (d - f). Original magnification, ×33.

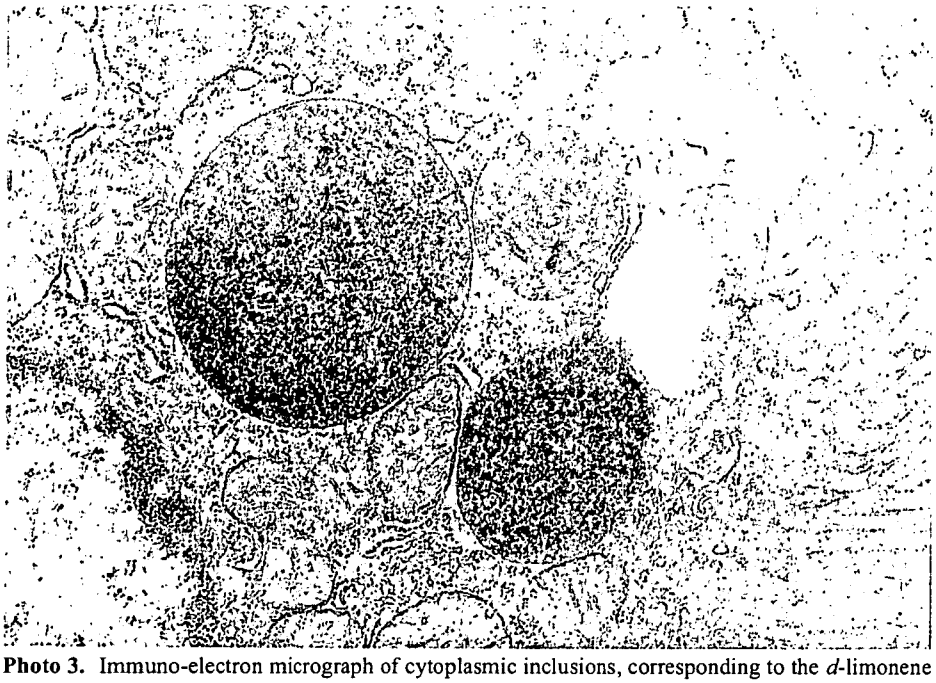

Photo 3. Immuno-electron micrograph of cytoplasmic inclusions, corresponding to the *d*-limonene induced hyaline droplets, in the epithelial cell of the renal proximal tubule. Colloidal gold particles are dispersed in the inclusions. Original magnification, ×10,000.

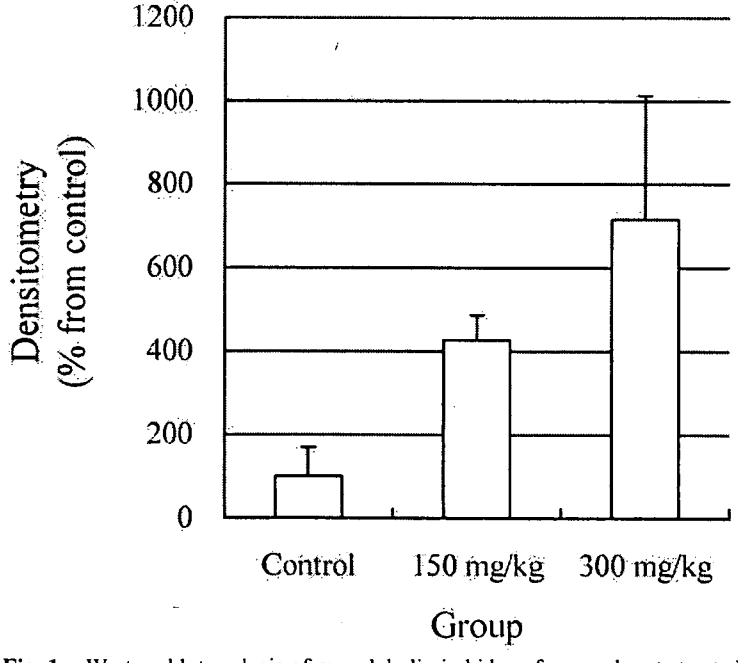

Fig. 1. Western blot analysis of α_{2u}-globulin in kidney from male rats treated with *d*-limonene.
Results are expressed as mean ± SD (n=4).

an alternative detection method, it is well known that α_{2u}-globulin droplets in the kidneys are negative for PAS reaction, but that they are stained positively by Azan-Mallory staining (U.S. EPA, 1991; Alden *et al.*, 1984). Although these additional stainings can distinguish hyaline droplets resulting from α_{2u}-globulin accumulation from those resulting from other causes, these analyses provide only indirect evidence. Direct evidence of α_{2u}-globulin accumulation in renal hyaline droplets could be required for appropriate risk assess-

Table 2. Grading results of histological/histochemical examination.

Chemical	Staining	Results		
		Control	Low dose	High dose
1,4-Dibromobenzene	HE [1]	−/−/±	++/++/+	++/+++/+++
	Azan-Mallory [2]	−/−/±	++/++/+	++/+++/+++
	Anti-α_{2u}-globulin [2]	−/−/±	++/++/+	++/+++/+++
Dicyclopentadiene	HE	−/−/−	+/++/++	+++/+++/+++
	Azan-Mallory	−/−/−	+/++/++	+++/+++/+++
	Anti-α_{2u}-globulin	−/−/−	+/++/++	+++/+++/+++
3,4-Dimethylaniline	HE	−/−/−	−/−/±	±/±/+
	Azan-Mallory	−/−/−	−/−/±	±/±/+
	Anti-α_{2u}-globulin	−/−/−	−/−/±	±/±/+
1,4-Dicyanobenzene	HE	−/−/−	±/+/+	++/+++/+++
	Azan-Mallory	−/−/−	±/++/+	+++/+++/+++
	Anti-α_{2u}-globulin	−/−/−	±/++/+	+++/+++/+++
Tetrahydrothiophene-1,1-dioxide	HE	+/−/−	+/+/++	++/++/++
	Azan-Mallory	+/−/−	++/+/++	++/++/++
	Anti-α_{2u}-globulin	+/−/−	++/+/++	++/++/++
1,3-Dicyanobenzene	HE	−/−/±	+/±/±	++/++/+++
	Azan-Mallory	−/±/±	+/±/±	++/+++/+++
	Anti-α_{2u}-globulin	−/±/±	+/±/±	++/+++/+++
Acenaphthene	HE	±/−/+	+/−/+	+/+/++
	Azan-Mallory	±/−/+	+/±/+	+/+/++
	Anti-α_{2u}-globulin	±/−/+	+/±/+	+/+/++
3,4-Dichloro-1-butene	HE	−/−/++	+/+/±	++/+/++
	Azan-Mallory	−/−/++	+/+/+	++/+/++
	Anti-α_{2u}-globulin	−/−/++	+/+/+	++/+/++
3a,4,7,7a-Tetrahydro-1*H*-indene	HE	+/+/++	++/++/++	+++/+++/+++
	Azan-Mallory	+/+/++	++/++/++	+++/+++/+++
	Anti-α_{2u}-globulin	+/+/++	++/++/++	+++/+++/+++
3,5,5-Trimethylhexan-1-ol	HE	−/−/±	+/+/++	+++/++/+++
	Azan-Mallory	±/−/±	+/+/++	+++/++/+++
	Anti-α_{2u}-globulin	±/−/±	+/+/++	+++/+++/+++
2,4-Di-tert-butylphenol	HE	−/−/−		−/−/−
	Azan-Mallory	−/−/−		−/−/−
	Anti-α_{2u}-globulin	−/−/−		−/−/−
4-Aminophenol	HE	−/±/−	−/−/−	−/−/−
	Azan-Mallory	−/±/−	−/−/−	−/−/−
	Anti-α_{2u}-globulin	−/±/−	−/−/−	−/−/−

[1] Grading for hyaline droplets.
[2] Grading for positive droplets.
No PAS-positive reaction for the hyaline droplets was observed in any sample.
Low dose for 2,4-di-*tert*-butylphenol was not examined.

ment, and a reliable detection method for the existence of α_{2u}-globulin is therefore necessary.

Using both immunochemical staining for paraffin-embedded sections and the immuno-electron microscopy technique, we demonstrated that our prepared antibody reacted specifically to α_{2u}-globulin in renal hyaline droplets in the male rats administered d-limonene, a well-known α_{2u}-globulin nephropathy inducer. The dose-dependent positive immuno-reaction of the antibody in both the tissue sections and the homogenates from d-limonene-treated rat kidneys indicated that the antibody could be applicable for semi-quantitative analysis. In addition, computational image analysis revealed that classical visual microscopic grading was also useful for semi-quantitative analysis of α_{2u}-globulin accumulation.

Although immunohistochemical α_{2u}-globulin analysis of the glycolmethacrylate-embedded sections had already been reported by Burnett *et al.* (1989), our method was advantageous from the standpoint of applicability to the paraffin-embedded sections. The paraffin-embedded specimens were usually prepared and stored for the general toxicity studies. In fact, all the sections used in experiment 2 in this study originated from study specimens which were prepared in the Japanese Existing Chemicals Survey Program conducted previously and stored for a long time. It indicated that our method is applicable to specimens derived directly from ordinary toxicology studies retrospectively. Hashimoto and Takaya (1992) previously investigated the application of α_{2u}-globulin immunostaining to paraffin sections by modifying the protocol of Burnett *et al.* (1989). The protocol includes pronase E treatment owing to enhancement of the antigen reactivity and removal of the non-specific reaction. Our method also includes the pronase E treatment, but

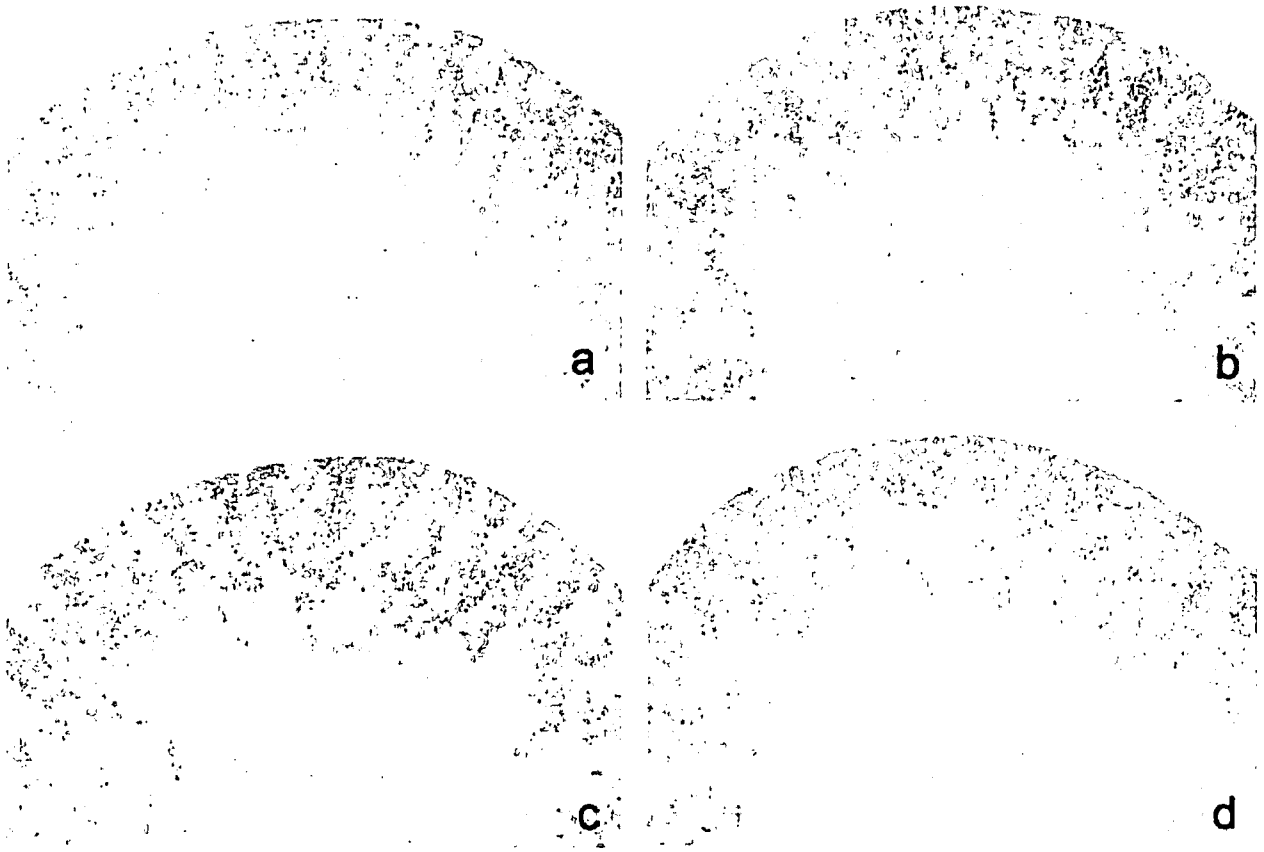

Photo 4. Immunohistochemical features of the anti-α_{2u}-globulin antibody, representing the four grades; minimal (a), slight (b), moderate (c) and severe (d). Original magnification, ×5.