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,c]	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,c]	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,c]	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 in Tween-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

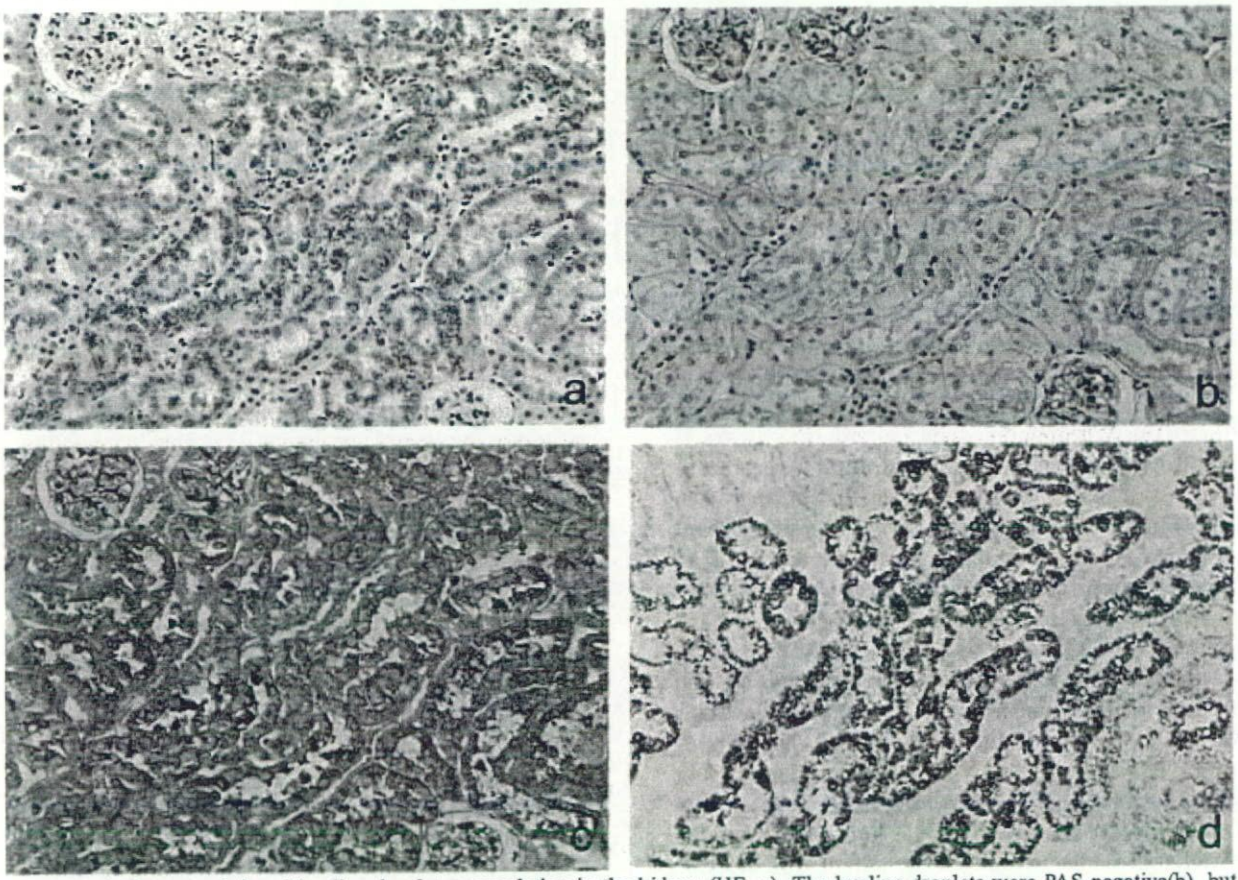

Photo 1. d-Limonene induced hyaline droplet accumulation in the kidney (HE, a). The hyaline droplets were PAS-negative(b), but they were stained positively with Azan-Mallory staining (c). Immunohistochemistry using the anti-α_{2u}-globulin antibody showed a clear positive reaction consistent with the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

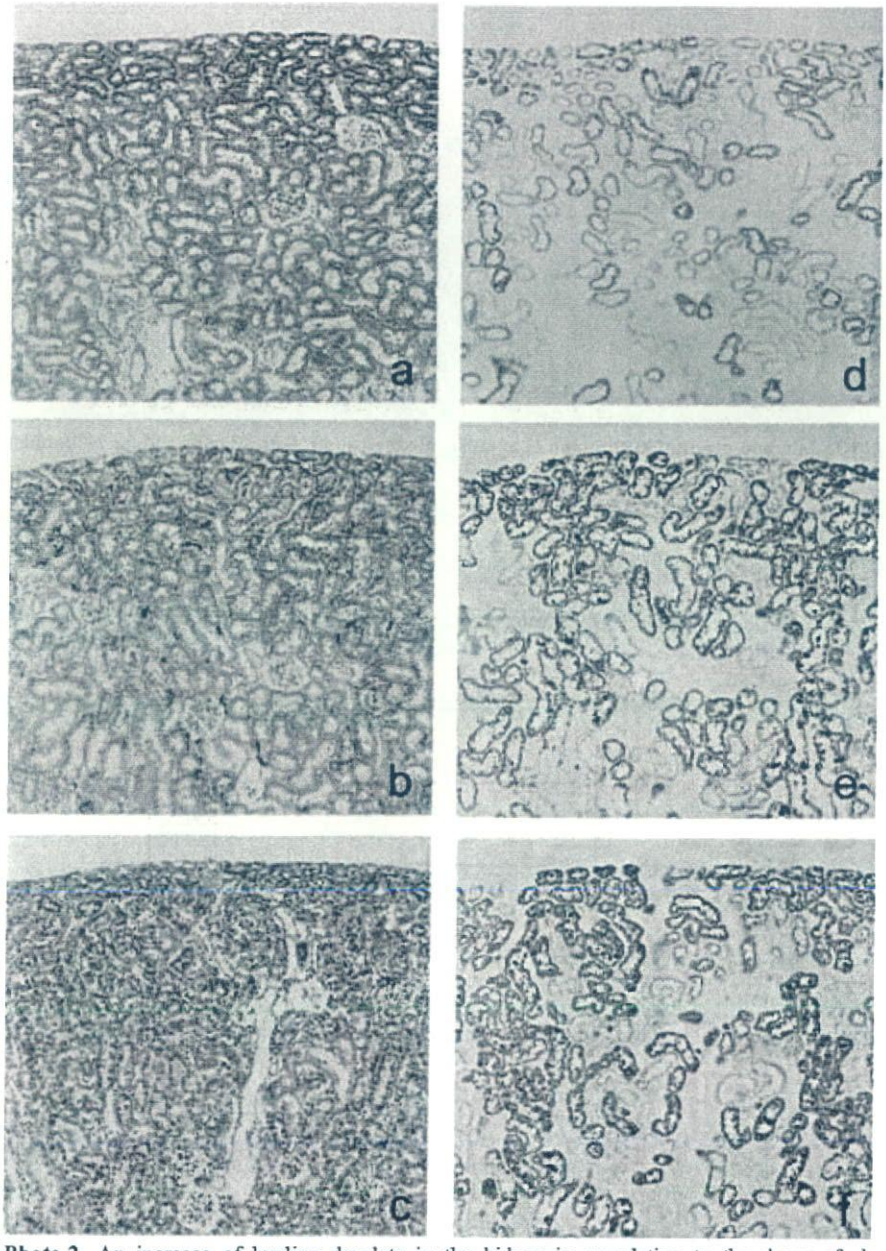

Photo 2. An increase of hyaline droplets in the kidney in correlation to the doses of *d*-limonene(HE, a - c). Positive reaction for the anti-α_{2u}-globulin antibody also increased with similar dose dependency (d - f). Original magnification, ×33.

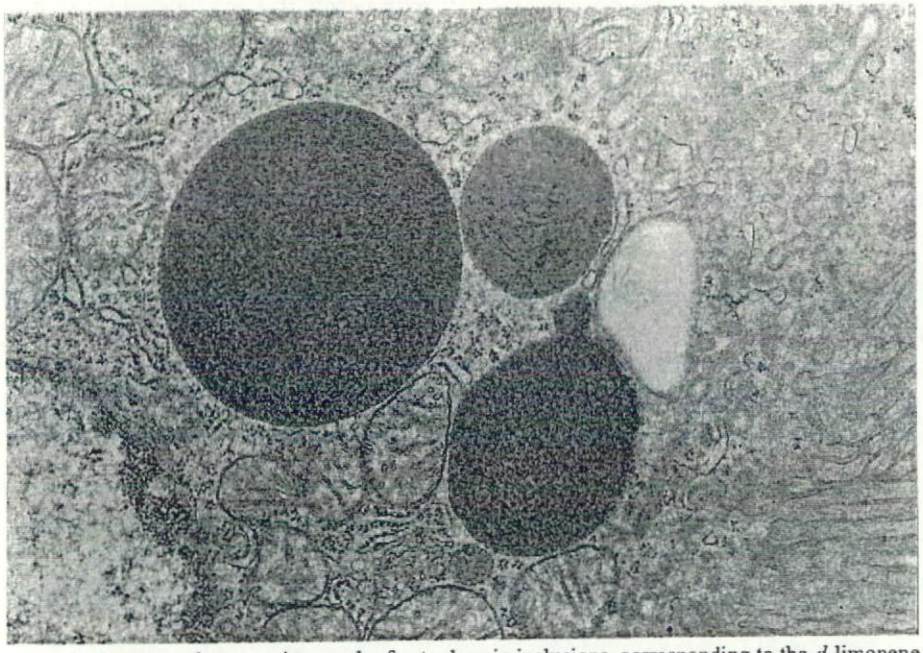

Photo 3. Immuno-electron micrograph of cytoplasmic inclusions, corresponding to the *d*-limonene induced hyaline droplets, in the epithelial cell of the renal proximal tubule. Colloidal gold particles are dispersed in the inclusions. Original magnification, ×10,000.

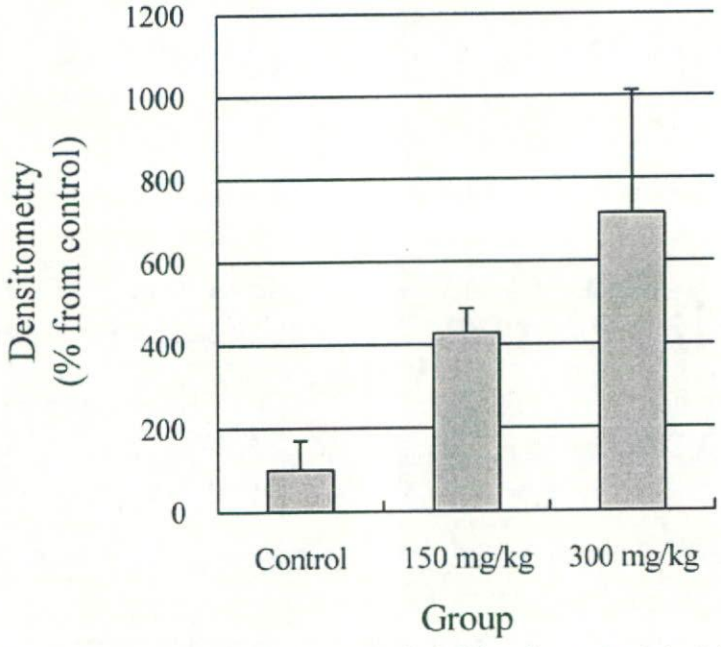

Fig. 1. Western blot analysis of α_{2u}-globulin in kidney from male rats treated with *d*-limonene.
Results are expressed as mean ± SD (n=4).

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

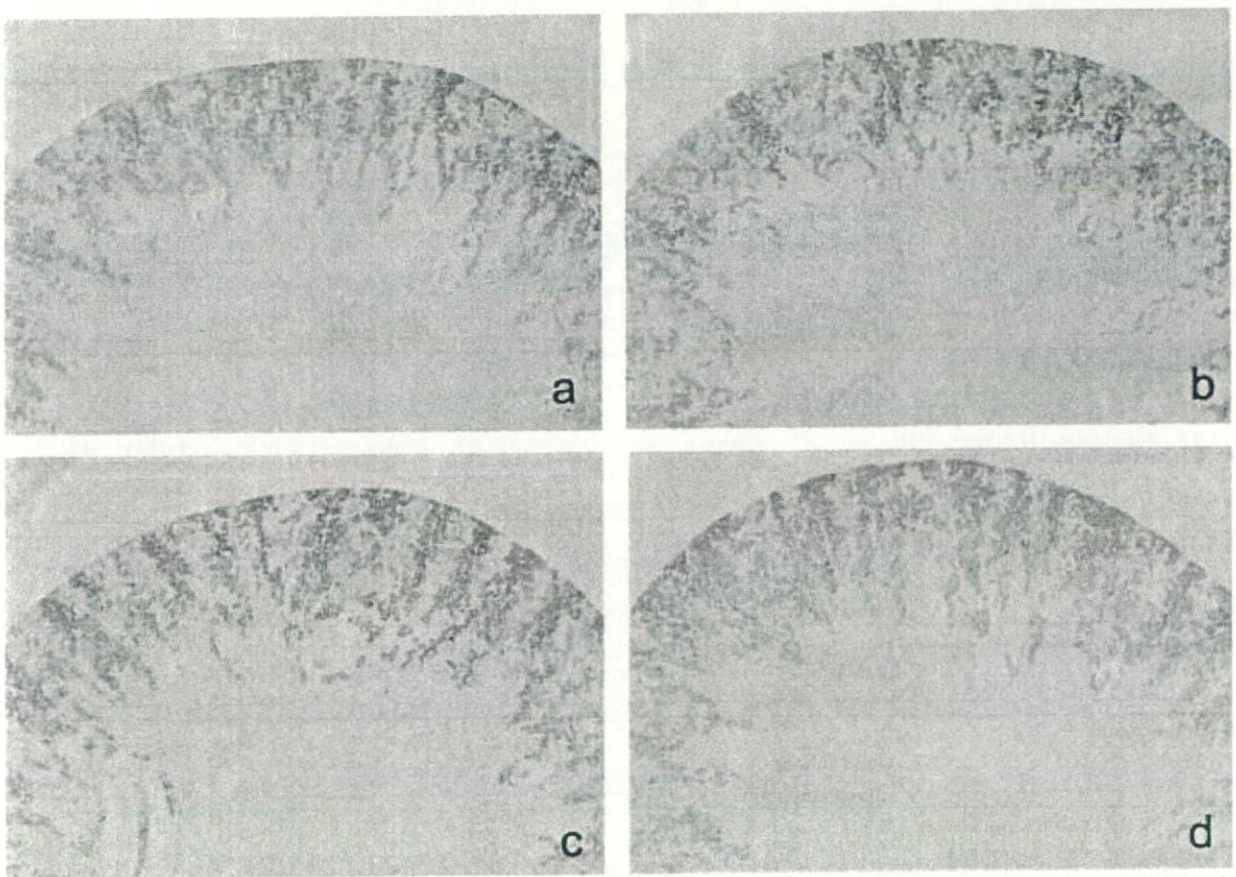

Photo 4. Immunohistochemical features of the anti-α_{2u}-globulin antibody, representing the four grades; minimal (a), slight (b), moderate (c) and severe (d). Original magnification, ×5.

Semi-quantitative immunohistochemical analysis of male rat-specific α_{2u}-globulin accumulation.

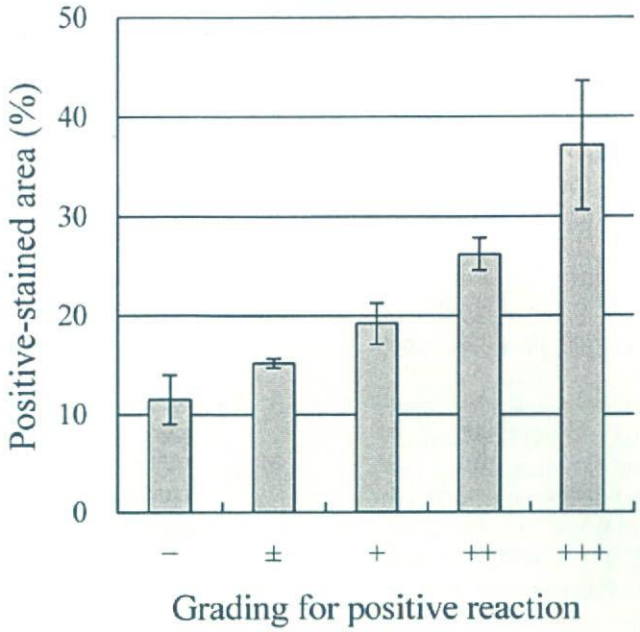

Fig. 2. Correlation between semi-quantitative and quantitative analyses for immuno-stained sections.
Results are expressed as mean ± SD (n=3).

the treatment is performed only in order to enhance the antigen activity and not to remove the non-specific reaction. This may suggest that our prepared antibody has a high specificity for α_{2u}-globulin. Caldwell *et al.* (1999) had conducted a similar quantitative immunohistochemical α_{2u}-globulin analysis, but it seems that the actual analyzed area was limited to narrower fields than in our study.

Urinary immunochemical analysis for detection of α_{2u}-globulin accumulation in male rat kidneys has been developed by Saito *et al.* (1996). Although the convenient urinary analysis is sufficient for detecting CIGA, the detectability is weaker than with kidney soluble protein analysis. The aim of the present analysis is not only to detect CIGA, but also to exclude the α_{2u}-globulin-induced nephrotoxic effects from risk assessment of chemicals. For 10 chemicals suspected of being CIGA, the occurrence of hyaline droplets in the kidneys with treatment was the lowest endpoint. In the process of evaluating chemical toxicity, if the most sensitive nephrotoxicity is concluded to be a neglected effect for human health, the NOAEL could be set based on other kinds of toxicological effects.

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Pediatric susceptibility to 18 industrial chemicals: A comparative analysis of newborn with young animals

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Abstract

We comprehensively re-analyzed the toxicity data for 18 industrial chemicals from repeated oral exposures in newborn and young rats, which were previously published. Two new toxicity endpoints specific to this comparative analysis were identified, the first, the presumed no observed adverse effect level (pNOAEL) was estimated based on results of both main and dose-finding studies, and the second, the presumed unequivocally toxic level (pUETL) was defined as a clear toxic dose giving similar severity in both newborn and young rats. Based on the analyses of both pNOAEL and pUETL ratios between the different ages, newborn rats demonstrated greater susceptibility (at most 8-fold) to nearly two thirds of these 18 chemicals (mostly phenolic substances), and less or nearly equal sensitivity to the other chemicals. Exceptionally one chemical only showed toxicity in newborn rats. In addition, Benchmark Dose Lower Bound (BMDL) estimates were calculated as an alternative endpoint. Most BMDLs were comparable to their corresponding pNOAELs and the overall correlation coefficient was 0.904. We discussed how our results can be incorporated into chemical risk assessment approaches to protect pediatric health from direct oral exposure to chemicals.

Keywords: Pediatric susceptibility; Industrial chemicals; Phenols; Newborn rats; Childhood exposure; Uncertainty factors; ADI; TDI; Benchmark dose

1. Introduction

Exposure of humans to environmental chemicals may occur via several routes such as the mouth, respiratory system, skin and eyes. As a result, regulatory/limit levels in food, water and air have been established to protect human health through risk assessment, which is usually based on toxicity data from animal studies (Hasegawa et al., 2004). However, the early postnatal period, especially the nursing phase, is not directly covered by current risk assessment approaches because of the inherent lack of toxicity information. Rather, two uncertainty factors are used to cover this data gap, one for human variability to toxic insult and the other for the lack of specific data to determine the critical effect (Dourson et al., 2002).

Repeated-dose oral rodent studies administer chemicals starting at approximately six weeks of age (OECD, 1995). In two-generation toxicity studies, chemicals are usually fed to rodents during the entire experimental period but newborn animals are only exposed to chemicals indirectly through maternal milk during nursing (up to 3 weeks old), or through small amounts of foods containing chemicals at about day 14 or older (OECD, 2001). Thus, there is generally no definitive toxicity information for chemical exposure in newborn animals.

Human infants may ingest not only baby foods and liquids but also household materials, fluids, and soil. They have unique physiological characteristics with regard to their organ/body balance, and the immature structure

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and functions of various organs may lead to elevated susceptibility or sensitivity (Scheuplein et al., 2002; Polin et al., 2004). Even though newborn exposure studies cannot be conducted for all chemicals due to ethical limitations, limited human and economic resources, or handling difficulties, such studies are valuable for the assessment of pediatric health risk given the appropriate comparative attention now being drawn to infant and child health world wide (Landrigan et al., 2004; IFCS, 2005).

Therefore, we have established an 18 day repeated-dose newborn rat toxicity study protocol, and conducted newborn studies for 18 industrial chemicals using this protocol, although the selected chemicals were mostly limited to phenolic compounds due to financial support. In addition, we have compared the newborn results with the results of a 28 day repeated-dose study (young study) and published all of the detailed analysis in peer-reviewed journals (Koizumi et al., 2001, 2002, 2003; Fukuda et al., 2004; Takahashi et al., 2004, 2006; Hasegawa et al., 2005; Hirata-Koizumi et al., 2005a,b).

In this article, we compare the results of these published studies by first describing our comparative study conditions common to all chemicals, then providing a summary of the final re-analyzed data, and finally discussing how our results can be incorporated into chemical risk assessment approaches to protect pediatric health from direct oral exposure to chemicals.

2. Experimental conditions of newborn and young studies for comparison

To appropriately elucidate differences in chemical sensitivity, studies in newborn and young rat were conducted under the same experimental conditions as closely as possible. For example,

(1) Sprague-Dawley SPF rats [Crj:CD(SD)IGS] purchased from Charles River Japan Inc. (Yokohama, Japan) were used for all studies;
(2) the same Lot Number for each chemical was used for both newborn and young studies;
(3) test solutions were prepared by the same methods with the same vehicles for both studies and administered by gastric intubation;
(4) test solutions were prepared at least once a week and kept cool and in the dark until dosing; stability was confirmed to be at least 7 days under these conditions; and
(5) all other reagents used in this study were specific purity grade;
(6) all animal treatments were conducted in 5 Japanese contract laboratories according to their Animal Care Guidelines and Japanese GLP Guidelines inspected by the Government.

The only differences in conditions were the administration period of 18 days for newborn and 28 days for young rats, and the recovery (maintenance) period as described in Fig. 1. Since rearing conditions for newborn rats change abruptly from nursing by foster mothers to individual self-feeding at postnatal Day 21 it was considered to be the best termination time point for the newborn dosing (a dosing period of 18 days) rather than adopting the same dosing period for the young studies (28 days).

2.1. Young studies

All schedules and examinations were performed in compliance with the Test Guideline "28 day repeated-dose toxicity study using mammals" of the Japanese Chemical Control Act (Official Name: Law Concerning the Examination and Regulation of Manufacture, etc. of Chemical Substances). This guideline is equivalent to OECD Test Guideline 407.

A dose-finding study was conducted according to the results of a single oral toxicity study. The study had a shorter dosing period (14 days) when compared to the main

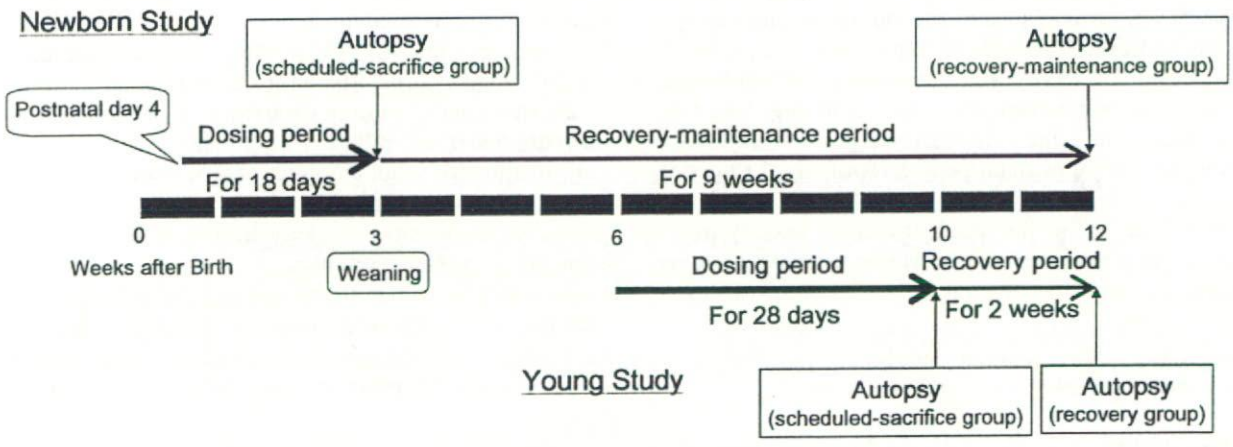

Fig. 1. Dynamic comparison of schedules for newborn and young studies.

study, and included most examination, but did not have examination of histopathology, urinalysis or recovery groups.

In the main study, at least 5 rats of both sexes were assigned to control, low, medium and high dose groups, and at least 5 rats of both sexes were assigned to control and high doses as recovery groups. Animals at 5–6 weeks of age were administered chemicals by gastric intubation daily for 28 days and sacrificed under ether anesthesia following the last treatment after overnight starvation (scheduled-sacrifice group). The recovery groups were maintained for 2 weeks without chemical treatment and sacrificed at 11 or 12 weeks of age. Observation of general behavior and estimation of body weight and food consumption were conducted during dosing and recovery periods. Macroscopic findings, blood chemistry (20 items), hematology (10 items), urinalysis (11 items), organ weights (15 organs) and histopathology (18 organs) were examined for the sacrificed animals.

2.2. Newborn studies

We established a newborn rat study protocol due to the lack of any standard test guideline for newborn animals. Fig. 1 shows the dosing and examination schedule for the newborn rat and young rat study. Pregnant rats (gestation day 14) were purchased and allowed to deliver spontaneously.

For a dose-finding study, all experimental conditions including the administration period were the same as the main study described below except that no examination of histopathology or urinalysis occurred, and no recovery-maintenance groups were maintained.

In the main study, dosing began on postnatal Day 4 with the administration of chemicals to 12 males and 12 female pups in each of 4 groups (control, low, medium and high doses). Each littermate consisted of 4 male and 4 female pups given different dose of chemical. Dosing to the pups continued up to weaning on postnatal Day 21 (18 days). On postnatal Day 22, half of the pups in each group were sacrificed under ether anesthesia (scheduled-sacrifice group), and remaining pups in all groups were maintained for 9 weeks without chemical treatment and subsequently sacrificed at 12 weeks of age (recovery-maintenance group). Observation of behavior and estimation of body weight and food consumption were conducted as with the young rat study protocol. The groups were examined for developmental parameters such as surface righting and visual placing reflexes for reflex ontogeny; fur appearance, incisor eruption and eye opening for external development during dosing period; and sexual development such as preputial separation, vaginal opening and estrous cycle during the recovery-maintenance period. The long recovery-maintenance period allowed for examination of sexual development after weaning and latent toxic effects in the early adulthood.

3. Unique approach to analysis of the susceptibility of newborn rats to chemicals

The no observed adverse effect level (NOAEL) is frequently used to determine safety or toxicity for environmental and industrial chemicals, with the NOAEL being the greatest dose at which no adverse effects are observed. However, the NOAEL is not always appropriate for an accurate comparison of toxicity levels between studies because the NOAEL is dependent on the dose setting. For example, in our early analysis of 2,4-dinitrophenol data, NOAELs for both newborn and young rat main studies were both 10 mg/kg/day because clinical signs of toxicity appeared at 20 mg/kg in newborn and 30 mg/kg in young rats. However, newborn rats seemed to be more sensitive to the chemical considering the intensity of lesions at higher doses. Further analysis of the data from the dose-finding young study showed no clinical toxicity signs at 20 mg/kg. Therefore, 20 mg/kg/day from the dose-finding young study was considered to be more appropriate as a NOAEL than the 10 mg/kg/day from the main young study. Including the dose-finding study in the determination of the NOAEL for a main study is not commonly done, thus, we decided to employ a new terminology in this document; the presumed NOAEL (pNOAEL) and defined it as the most likely no adverse effect dose for our specific purpose. The lack of information from dose-finding studies, such as histopathological examination in both newborn and young studies, and the shorter administration period in the young case was carefully considered in adopting the pNOAEL approach.

In addition, a Benchmark Dose (BMD) approach was applied to the same toxicity endpoint data that was used for the estimation of pNOAEL. Although clinical signs and histopathological changes are generally not appropriate for BMD analysis, since the frequency, duration and severity cannot usually be incorporated for the calculation, we attempted to employ incidences such as numbers of affected animals from both main and dose-finding studies where appropriate. Using the US EPA provided Benchmark Dose Software (Version 1.3.2), Benchmark Dose Lower Bound (BMDL) was estimated with 10% extra incidence at the 95% confidence level. In most cases, the incidence data were input to a Dichotomous model. For selection of the model, the lowest AIC (Akaike's Information Criterion) was used and the goodness-of-fit was confirmed visually with graphical displays.

At the first trial to evaluate the susceptibility of newborn rats to chemicals, we judged that the above endpoint comparison of pNOAEL/BMDL was not sufficient with respect of outcome reliability and the full toxicity data set should have been used. Alternatively, comparison of pNOAEL/BMDL might be sufficient for low dose responses but not with results at Lowest Observed Adverse Effect Level (LOAEL). In fact, it is reported that 17-day-old rats show higher susceptibility to chlorpyrifos at the maximum tolerated dose than adult rats (Moser and Padilla, 1998),