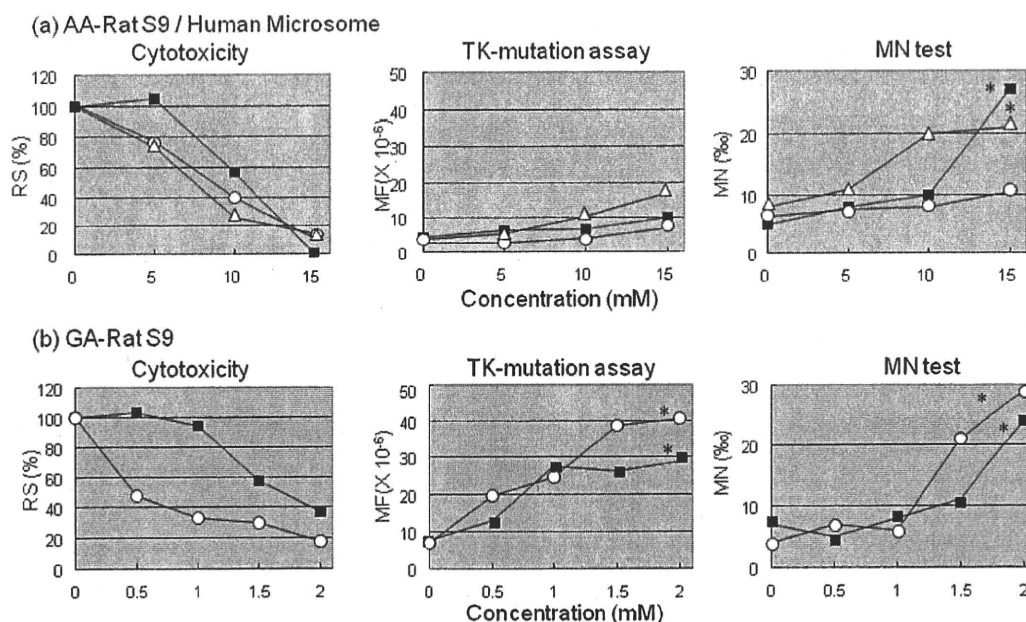

Fig. 1. Cytotoxic (relative survival, RS) and genotoxic (TK and MN assays) responses of TK6 cells treated with AA or GA for 4 hr with or without metabolic activation. (**a**) TK6 cells were treated with AA without (■) or with (○) rat liver S9 or human microsomes (△). (**b**) TK6 cells were treated with GA without (■) or with (○) rat liver S9. $*P < 0.05$ (Omori method for TK-mutation assay, trend test for MN assay).

We also seeded cells into the 96-well plates in the absence of TFT to determine plating efficiency (PE3). TK6 cells were seeded at 40,000 cells/well and 1.6 cell/well for TFT and PE plates, respectively. AHH-1 and h2E1v2 cells were seeded at 5,000 cells/well and 3.2 cells/well for TFT and PE plates, respectively. All plates were incubated at 37°C in 5% CO_2 in a humidified incubator. We scored for the colonies in the PE plates at 14th day after plating, and scored for the colonies in the TFT plate on the 28th day after plating. Mutation frequencies were calculated according to the Poisson distribution [Furth et al., 1981]. The data were statistically analyzed by Omori's method, which consists of a modified Dunnett's procedure for identifying clear negative, a Simpson-Margolin procedure for detecting downturn data, and a trend test to evaluate the dose-dependency [Omori et al., 2002]. We evaluated cytotoxicity for TK6 by relative survival (RS), which is calculated from plating efficiency (PE0), and for AHH-1 and h2E1v2 by relative suspension growth (RSG), which is calculated from cell growth rate during 3 days expression period.

Western Blot Analysis

A goat polyclonal anti-rat CYP2E1 antibody (Daiichi Pure Chemical, Tokyo) and rabbit anti-rat actin (Sigma, St. Louis, MO) were used as primary antibodies. AP-conjugated secondary antibody (Cappel, Organon Technika Corp., West Chester, PA) was used to detect primary antibody signals.

DNA Adduct Assay

As a standard for LC/MS/MS analysis, N7-GA-Gua and [$^{15}N_5$]-labeled N7-GA-Gua were synthesized as described previously [Gamboa da Costa et al., 2003]. DNA was extracted from the cells by using DNeasy 96 Blood &Tissue Kit (QIAGEN, Düsseldorf) and incubated at 37°C for 48 hr for deprination. An aliquot of the [$^{15}N_5$]-labeled N7-GA-Gua standard was added to each sample and filtered through an ultrafiltration membrane to remove DNA. The eluted-solution was evaporated thoroughly and dissolved in water, and then the solutions were subsequently quantified by LC/MS/MS.

RESULTS

Cytotoxicity and Genotoxicity of AA and GA Under Metabolic Activation

We used human microsomal preparation and phenobarbital- and 5,6-benzoflavone-treated rat liver S9 for metabolic activation. CYP2E1 activity of the human microsomal preparation was more than twice that of the rat liver S9 preparations (2,917 vs. 1,295 pmol/mg/min).

Figure 1 shows the cytotoxicity (RS; relative survival), MN, and *TK*-mutations induced by AA (a) and GA (b) with and without rat liver S9 or human microsomes. Rat liver S9 or human microsomes enhanced cytotoxicity (RS) of AA and GA. On the other hand, AA showed weak genotoxicity only at relatively high concentrations (>10 mM) without S9, but neither activating system enhanced the weak genotoxicty. GA induced TK-mutations dose-dependently from the low concentration (0.5 mM) and induced MN from 1.5 mM both with and without S9. Thus, neither the rat nor human metabolizing system activated AA or inhibited the expression of GA genotoxicity.

umu Assay Using Strains Expressing Human CYP2E1

We used *S. typhimurium* OY1002/2E1 strain to assess the cell toxicity and genotoxicity of AA at exposures up to 10mM (Fig. 2c). We also examined AA and GA with

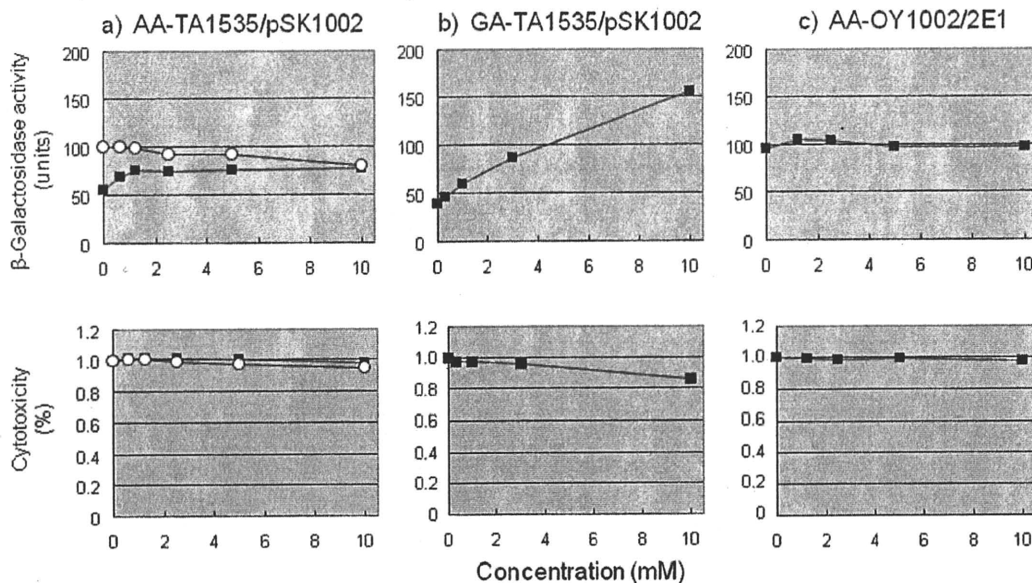

Fig. 2. Induction of *umuC* gene expression and cytotoxic response by AA (**a**, **c**) or GA (**b**) in *S. typhimurium* tester strains TA1535/pSK1002 (a, b) and OY1002/2E1 (c). The *umu* tests were conducted without (■) or with rat S9 (○). β-Galactosidase activity (units) was determined as described in Materials and Methods. Cytotoxic activities are expressed as % optical density change at 600 nm.

or without rat S9 using TA1535/pSK1002 strain. Although GA clearly produced a dose-related increase in response to DNA damage (Fig. 2b), AA elicited no genotoxic or cell toxic response with and without S9 (Fig. 2a). Thus, we could not demonstrate any in vitro genotoxicity of AA in the bacterial system.

Cytotoxic and Genotoxic Responses to AA in Transgenic Cell Lines

Western blot analysis revealed that h2E1v2 accumulated more CYP2E1 than either of its parental cell lines (Fig. 3). Both the h2E1v2 and AHH-1 cells exhibited weak responses (TK-gene mutations and MN) to AA at ≤3 mM with little difference in cytotoxicity (RSG, relative suspension growth) (Fig. 4a). h2E1v2 differed from AHH-1, however, in that it showed clear genotoxic and cytotoxic responses (RSG) to DMN, which is a representative substrate for CYP2E1 (Fig. 4b). Thus, the h2E1v2 cell line had CYP2E1 activity but did not activate AA.

DNA Adduct Formation by AA and GA in the Cell Lines

AA induced trace amounts of N7-GA-Gua adduct in TK6 cells (with and without S9) (Fig. 5a) and in AHH-1 and h2E1v2 cells (Fig. 5b). GA, on the other hand, induced a substantial number of N7-GA-Gua adducts in TK6 cells (Fig. 5c). These results suggest that the expression of genotoxicity may be dependent on N7-GA-Gua adduct formation, and the in vitro metabolic activation system did not metabolize AA into GA.

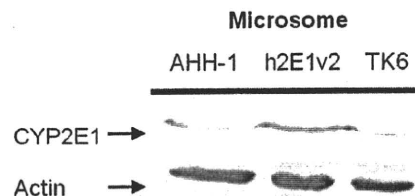

Fig. 3. Western blot analysis of CYP2E1 in AHH-1, h2E1v2, and TK6 cells. Equal amount of materials were loaded for each sample. CYP2E1 protein was stained with the anti-CYP2E1 antibody. Actin was used as a loading control.

DISCUSSION

A large number of studies about the in vitro genotoxicity of AA have been reported [Dearfield et al., 1995; Besaratinia and Pfeifer, 2005]. AA was negative in Ames assay in both the presence and absence of S9 [Zeiger et al., 1987; Knaap et al., 1988; Tsuda et al., 1993]. In mammalian cell assays, cytogenetic tests such as chromosome aberration test and sister chromatid exchange tests were positive [Sofuni et al., 1985; Tsuda et al., 1993]. AA also induced *Tk* mutation in the MLA but did not induce *Hprt* mutation in V79 cells [Moore et al., 1987;

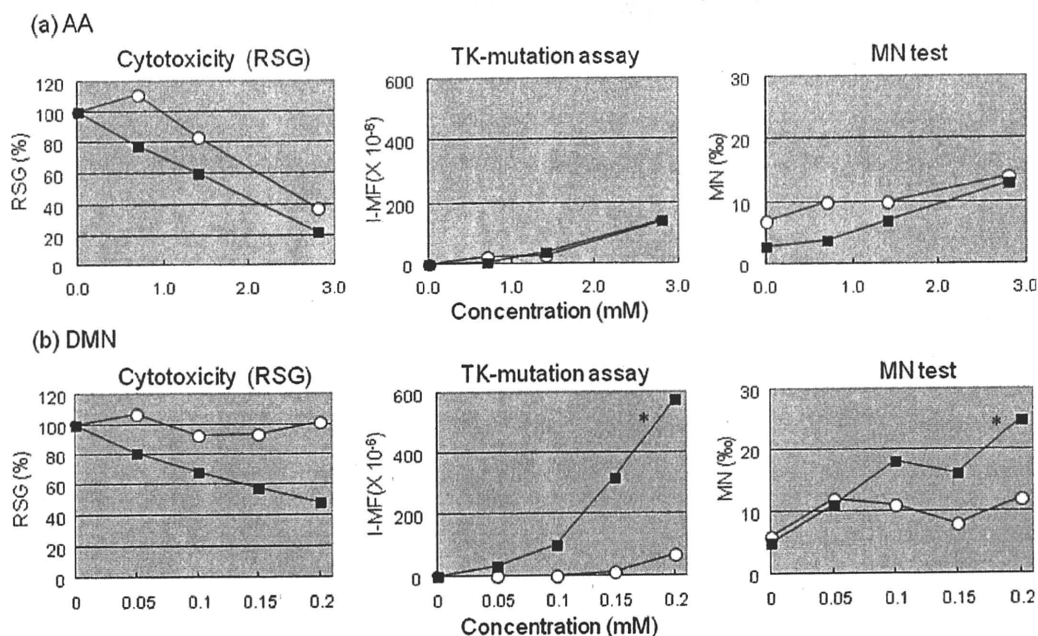

Fig. 4. Cytotoxic (relative suspension growth, RSG) and genotoxic (TK assay and MN test) responses of AHH-1 (○) or h2E1v2 (■) cells treated with AA or DMN for 4 hr. I-MF means induced mutation fraction, in which back ground mutation frequency is subtracted. $^*P < 0.05$ (Omori method for TK-mutation assay, trend test for MN assay).

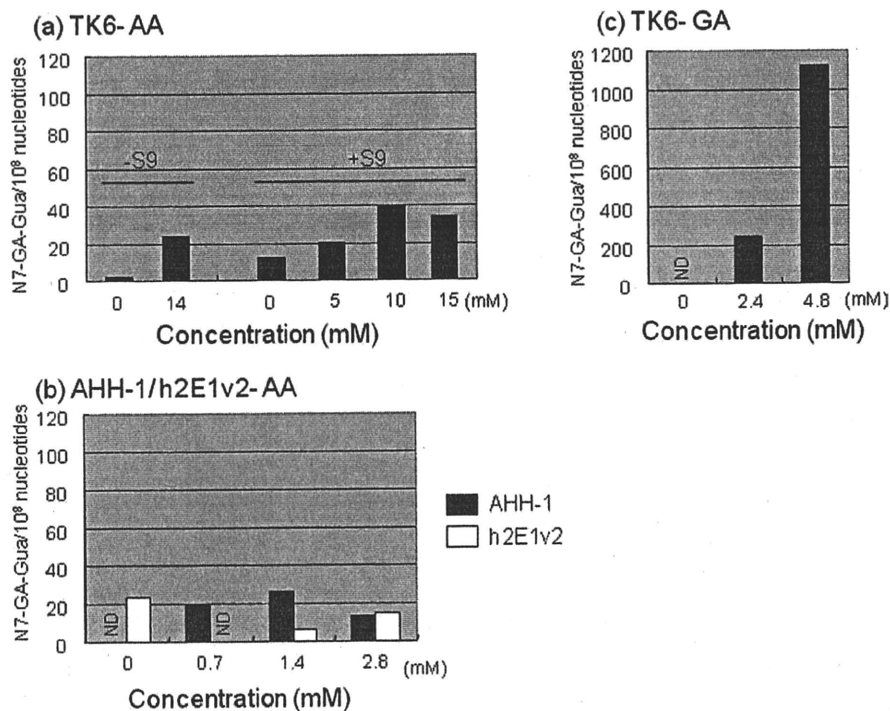

Fig. 5. Levels of N7-GA-Gua adduct in TK6 (**a**, **c**), AHH-1 (**b**), or h2E1v2 (b) cells treated with AA (a, b) or GA (c) for 4 hr at different concentrations. Data are expressed as the number of adducts in 10^8 nucleotides.

Knaap et al., 1988; Tsuda et al., 1993; Baum et al., 2005; Mei et al., 2008], and produced negative results in the Comet assay with V79 cells and human lymphocytes [Baum et al., 2005]. We also obtained positive results in *TK* gene mutation and micronuclei assays, but not in Comet assay using human lymphoblastoid TK6 cell in the absence of S9 [Koyama et al., 2006]. To obtain the positive results in the MLA and TK6 cells, however required very high dose of AA, which was sometimes beyond the top dose of the OECD testing guideline (>10 mM) [Koyama et al., 2006; Mei et al., 2008]. The spectrum of AA-induced *TK* mutations in TK6 and *cII* mutations in Big Blue® mouse embryonic fibroblasts were not significantly different from the spontaneous one, although its metabolite GA distinctly induced a specific point mutation [Besaratinia and Pfeifer, 2003, 2004; Koyama et al., 2006]. Thus, the in vitro genotoxicity of AA is still controversial.

In contrast, the in vivo genotoxicity of AA has been clearly demonstrated by various rodent genotoxicity tests including micronuclei tests in peripheral blood [Cao et al., 1993; Abramsson-Zetterberg, 2003; Manjanatha et al., 2005], transgenic gene mutation in liver [Manjanatha et al., 2005], and Comet assay in various organs [Ghanayem et al., 2005b]. AA has also proven to be genotoxic to germ cells [Dearfield et al., 1995]. AA induced micronuclei in mice spermatids, and heritable chromosome translocations and specific locus mutations in postomeiotic sperm and spermatogonia [Lahdetie et al., 1994; Xiao and Tates, 1994]. AA also elevated the frequency of dominant lethal mutations probably accompanying with chromosome aberrations leading to death of embryo [Shelby et al., 1987; Adler et al., 1994]. The International Agency for Research on Cancer (IARC) classified it as 2A, a probable human carcinogen based on finding of rodent carcinogenicity [IARC, 1994]. AA caused tumors in various organs including mammary gland, peritesticular mesothelium, thyroid, and central nervous system [Carere, 2006], although the AA-inducing genotoxicity in these organs have not been demonstrated.

AA is metabolized either via direct glutathione conjugation followed by excretion of mercapturic acid or via oxidative pathways catalyzed by CYP2E1 to yield GA [Calleman et al., 1990; Wu et al., 1993; Sumner et al., 1999]. GA reacts quickly with DNA, mainly forming N7-GA-Gua adduct. Genotoxicity of GA has been demonstrated in vitro and in vivo. In contrast to AA, GA is positive in most genotoxicity tests [Hashimoto and Tanii, 1985; Dearfield et al., 1995; Besaratinia and Pfeifer, 2004; Baum et al., 2005; Koyama et al., 2006]. Manjanatha et al. [2005] demonstrated in transgenic Big Blue® mice that both AA and GA induces endogenous *Hprt* and transgenic *cII* mutation at same level, and also produced similar mutational spectra. The predominant type of mutations observed in these two systems was G:C to T:A transversion, which is presumably derived from N7-GA-Gua [Besaratinia and Pfeifer, 2005]. The in vivo results with transgenic Big Blue® mice indicate that in vivo expression of AA genotoxicity is mediated via its GA metabolite.

However, no one has succeeded in demonstrating metabolically activated AA genotoxicity in vitro [Knaap et al., 1988; Tsuda et al., 1993; Dearfield et al., 1995; Friedman, 2003; Emmert et al., 2006]. In this study, we used induced rat liver S9 and human microsomal fraction for the metabolic activation. Although they have high CYP2E1 activity, the AA-inducing genotoxicity was never influenced by the presence of the exogenous metabolic activation system (Fig. 1a). We assumed that GA, a reactive epoxide, could be rapidly inactivated through microsomal epoxide hydrolase or glutathione in any S9 or microsomal fraction resulting in either the metabolism or the conjugation and detoxification of GA [Sumner et al., 2003; Decker et al., 2009]. However, presence of rat S9 did not prevent GA from inducing *TK*-mutation and micronuclei.

The *umu* assay could not detect the genotoxicity of AA even by the strain (Fig. 2). Emmert et al. [2006] also failed to demonstrate the mutagenicity of AA in the Ames test using the metabolically competent *S. typhimurium strain* YG7108pinERb_5 that expresses CYP2E1. In mammalian cell system, such as the human lymphoblastoid cell line, h2E1v2 overexpressing human CYP2E1 did not show different response in *TK*-gene mutation and MN induction compared to its parental cell line, AHH-1, although these cell lines exhibited distinct difference to DMN, which is a representative substrate for CYP2E1. We also investigated the genotoxicity of AA in h2E1v2 cells after long exposure (24 hr), because AA may be slowly metabolized to GA. The result was also negative (data not shown). Thus, we could not obtain any evidence of in vitro genotoxicity of AA *via* metabolic activation.

Glatt et al. [2005] developed a Chinese hamster V79-derived cell line that stably expresses human CYP2E1 and sulphotransferase (SULT), and applied it to investigate sister chromatid exchanges (SCE) induced by some chemicals. They demonstrated that AA induced SCE in the transgenic cell line but not in the parental line. Although the reason for the discrepancy between their results and ours is not clear, it is possible that another enzyme, such as SULT, may be involved in metabolic activation of AA.

The DNA adduct analysis clearly revealed that h2E1v2 cells does not generate N7-GA-Gua adduct in vitro. Because exposure of human cells to GA results in significant accumulation of N7-GA-Gua adduct, but DNA adduct analysis following exposure of h2E1v2 with AA does not generate N7-GA-Gua adduct in vitro, lead one a conclusion that the presence of CYP2E1 alone is not enough to metabolize AA to GA in mammalian cells. The

DNA adduct analysis also strongly supports a hypothesis that GA contribute to its genotoxicity by forming N7-GA-Gua adduct. Interestingly, very small amount of N7-GA-Gua adduct was generated in TK6 cells in a dose-dependent manner regardless of the presence of S9 (Fig. 5a). TK6 cells themselves may have an enzymatic activity to metabolize AA to GA, although its activity must be extremely low. Ghanayem et al. [2005b] showed that AA was not mutagenic or genotoxic in CYP2E1-null mice. Intraperitoneal injection of AA (25, 50 mg/kg) by once daily for 5 days induced micronuclei in erythrocyte and DNA damage assessed by Comet assay in leukocyte and liver cells of wild-type, but not in the CYP2E1-null mice. The plasma concentration of AA in the CYP2E1-null mice was 115-times higher than in the wild-type mice, while the GA concentration in the CYP2E1-null mice was negligible compared to that in the wild-type mice [Ghanayem et al., 2000]. Ghanayem et al. [2005c] also demonstrated that AA produces dominant lethal in mice that express CYP2E1, but not in mice that do not express CYP2E1, indicating that induction of germ cell mutations by AA in mice in vivo is also dependent upon CYP2E1 metabolism. These results clearly suggest that CYP2E1 is the principal enzyme responsible for the metabolism of AA to GA in vivo.

In conclusion, AA could not be metabolized to GA by in vitro metabolic activation system commonly used in genotoxicity tests. In vivo, on the other hand, GA is apparently responsible for AA-inducing genotoxicity. Although AA may exhibit genotoxicity in in vitro mammalian cells at high concentrations, its positive response is not relevant for its major genotoxicity. AA could be classified into in vivo specific genotoxic chemical.

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ORGAN TOXICITY AND MECHANISMS

Life stage-related differences in susceptibility to acrylamide-induced neural and testicular toxicity

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Abstract In order to assess age-dependence of susceptibility to acrylamide (ACR)-induced neural and testicular toxicity, 3- and 7-week-old male SD rats were given ACR at 0, 50, 100, or 200 ppm in the drinking water for 4 weeks, and the nervous and male reproductive systems were examined histopathologically. Testicular genotoxicity was evaluated with the comet assay and the micronucleus (MN) test. Glutathione *S*-transferase (GST) activity and glutathione (GSH) content in the liver and testis were also measured. In both young and adult animals, neurotoxicity was evident from 100 ppm and increased in proportion to ACR intake per body weight. In the testis, marked degeneration and exfoliation, mainly of spermatids, were observed from 100 ppm limited to young animals. The comet assay revealed ACR to significantly induce DNA damage from 100 ppm in both life stages, while MNs were found only in young rats from 100 ppm. The level of GST activity in the testis of young rats at the end of experiment was significantly lower than that of adult animals, regardless of the ACR treatment. There were no life stage-related differences in GSH contents in the liver and testis. These results suggest that susceptibility to neurotoxicity might not differ between young and adult rats when exposure levels are adjusted for body weight. Regarding testicular toxicity, young animals around puberty proved more susceptible than adult animals, possibly due to their lower level of testicular GST activity than that in adult animals.

Keywords Acrylamide · Age · Susceptibility · Neurotoxicity · Testicular toxicity · Rat

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Introduction

Acrylamide (ACR), a proven carcinogen in animals known to be a neuro- and reproductive toxicant, has found many industrial and laboratory uses and therefore is a potential hazard to man. Recently, it was found to be formed on heating from carbohydrate and amino acid components of food, so that public exposure has become a worldwide concern (Exon 2006; Parzefall 2008). Mean daily intake of ACR for adults is estimated as 1 μg/kg body weight/day, but values for infants and children are estimated to be two- to threefold higher when expressed on a body weight basis (WHO/IPCS 2006). So far, since toxicity studies of ACR have mainly been performed using adult animals, to evaluate toxicity targeting children is important for risk assessment of ACR exposure in man.

Even the limited studies on susceptibility to neurotoxicity of ACR in relation with the life stage have not generated consistent results controversial. For example, whereas a rotarod test revealed slightly higher sensitivity with adult when compared to immature rats regarding disruption of locomotor activity (Kaplan and Murphy 1972), earlier onset of neurological symptoms as well as faster induction of severe myelin disruption was found with

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suckling rats given ACR at 50 mg/kg by intraperitoneal injection 3 times weekly when compared with adult rats (Suzuki and Pfaff 1973). Similarly, mice given ACR at 400 ppm in the drinking water revealed earlier onset of neurological symptoms and terminal nerve swelling by administration starting from 3 weeks of age than from 8-weeks of age (Ko et al. 1999).

As for testicular toxicity, no data on the susceptibility to ACR in relation with testicular development have been reported. In our previous study, although retardation of spermatogenesis was observed in preweaning rats exposed to ACR via maternal drinking water or direct intraperitoneal injection, no histopathological changes suggestive of testicular toxicity were noted (Takahashi et al. 2009). Therefore, testes prior to spermatogenesis could be less sensitive to ACR.

In the present study, to elucidate factors influencing susceptibility to ACR-induced neuro- or testicular toxicity, we compared histopathological changes of the nervous and male reproductive systems between rats exposed to ACR during the puberty growth period and the young adult stage. The comet assay and micronucleus (MN) test were also conducted to evaluate testicular genotoxicity induced by ACR. Additionally, to compare the capacity for ACR detoxification between the young and adult animals, glutathione *S*-transferase (GST) activity and glutathione (GSH) content were measured in the liver and testis.

Materials and methods

Experimental design

Thirteen pregnant Crj:CD (SD) rats were obtained from Charles River Japan Inc. (Yokohama, Japan) at gestational day 10. They were housed individually in polycarbonate cages with wood chip bedding and maintained in an air-conditioned animal room (temperature: 24 ± 1°C, relative humidity: 55 ± 5%, 12-h light/dark cycle) with basal diet (CRF-1, Oriental Yeast Co., Tokyo, Japan) and tap water available ad libitum. After delivery, 40 male pups on weaning at 21 days of age were allocated to 4 groups, each consisting 10 animals from different dams, given ACR (Sigma, St. Louis, MO, USA; CAS #79-06-1) at 0, 50, 100, and 200 ppm in the drinking water for 4 weeks (**young group**). Similarly, forty male SD rats at 6 weeks of age were obtained from Charles River Japan Inc., and acclimatized with basal diet (CRF-1) and tap water ad libitum for 1 week. Then, they were randomly divided into 4 groups and given ACR at 0, 50, 100, and 200 ppm in the drinking water for 4 weeks (**adult group**). The highest dose was set as the dose that induces neurotoxicity and testicular toxicity within 4 weeks in adult male rats (Lee et al. 2005). Observations for clinical signs and mortality were conducted daily. Body weights and food consumption were recorded every week. In addition, animals were scored with respect to the appearance of gait abnormalities, as previously reported (Moser 1991; Shell et al. 1992; Lee et al. 2005), as follows: grade 1, normal gait; grade 2, slightly abnormal gait with slight degrees of ataxia, hopping gait, and foot splay; grade 3, moderately abnormal gait with moderate degrees of ataxia, foot splay, and limb abduction; grade 4, severely affected gait, including inability to support the body weight as well as foot splay.

At necropsy, all animals were killed under deep anesthesia by exsanguination, and the brain, testes, and epididymides were removed and weighed. The trigeminal nerve was also removed. The brains from 5 animals per each group were fixed in methacarn solution at 4°C overnight. The brains from another 5 rats per each group, along with the trigeminal nerves and epididymides were fixed in neutrally buffered 10% formalin. One testis was used for comet assays, and the other was fixed in Bouin's solution at room temperature overnight. All fixed tissues were routinely processed for paraffin embedding, sectioned at 3 μm, and stained with hematoxylin and eosin (HE). The sciatic nerves were exposed at autopsy and subjected to in situ fixation by immersion in ice-cold 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 min (Takahashi et al. 2009). The portion located at the ankle position was carefully dissected and further fixed with fresh fixative overnight, postfixed in 1% osmium tetroxide (TAAB Laboratories Equipment Ltd., Berkshire, UK) in the same buffer for 2 h at 4°C, and embedded in epoxy resin (TAAB Laboratories Equipment Ltd.). Semithin sections, 1 μm in thickness, were stained with toluidine blue for light microscopic assessment.

As a satellite study, 3- or 7-week-old male SD rats were similarly given ACR at 0 or 200 ppm in the drinking water for 4 weeks. The livers and unilateral testes were collected for measurement of GST activity and GSH content. Small portions of each testis were fixed in Bouin's solution to confirm ACR-induced lesions microscopically.

The animal protocol was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

Morphometric assessment

To evaluate aberrant dot-like structures immunoreactive with synaptophysin (SYP) in the cerebellar molecular layer, methacarn-fixed cerebellum sections were subjected to immunohistochemistry for SYP, as described previously (Takahashi et al. 2008), with rabbit polyclonal antibody Ab-4 (1: 200, Lab Vision Corp., Fremont, CA, USA) as the primary antibody.

For morphometry of axonal degeneration in the sciatic nerves and SYP-immunoreactive aberrant dot-like structures in the cerebellar molecular layer, photomicrographs were taken with a digital camera attached to a microscope (DP71, Olympus Corp., Tokyo, Japan). Measurement was then performed using image analysis software (WinROOF, Version 5.7.1, Mitani Corp. Tokyo, Japan). The total number of axons/unit area and the numbers of degenerated axons and the diameters of axons were assessed in one cross-sectional area at 400× magnification of toluidine blue-stained specimens from each animal, and the density, percentage of degenerated axons, and percentage of myelinated axons less than 3 μm in diameter were calculated. For evaluation of SYP-immunoreactive aberrant dot-like structures, numbers of dots in the left cerebellar hemisphere were counted following measurement of the length of the cortex in one cross-sectional area at 12.5× magnification and the number of SYP-immunoreactive dots/unit length of the cortex was calculated.

For testicular toxicity, approximately 400–650 circularly sectioned seminiferous tubules for each rat were assessed microscopically, and then the percentages of tubules with histopathological changes were calculated.

Comet assay

The procedures for preparing and processing comet assays were performed according to the recommendation by an expert working group on the comet assay in the International Workshop on Genotoxicity Testing (IWGT) (Tice et al. 2000; Burlinson et al. 2007) and slightly modified for testes. Briefly, each testis was washed with cold mincing/homogenizing buffer containing Hanks' Balanced Salt (HBSS) Solutions (Invitrogen Corporation, Carlsbad, CA, USA), 20 mM EDTA·2Na, and 10% DMSO, minced with scissors, and placed on ice for 15–30 s to precipitate clumps of cells. The supernatant was suspended in 0.5% Nusieve GTG agarose (Lonza, Basel, Switzerland), quickly layered on a MAS-coated slide (Matsunami Glass Ind. Ltd., Osaka, Japan), immersed in lysing solution (pH10, 100 mM EDTA·2Na, 2.5 M NaCl, 10 mM Tris(hydroxymethyl)aminomethane containing 1% Triton-X and 10% DMSO) at 4°C overnight, and electrophoresed for 15 min in alkaline buffer after the unwinding treatment. Then, the cells were fixed with ethanol and stained with SYBER green (Molecular Probes, Eugene, OR, USA) according to the manufacturer's recommendation. We observed the cells under a fluorescence microscope (BX50, Olympus Co.). Round-shaped cells, considered as spermatocytes and early spermatids, were captured with a CCD camera. At least 100 cells were observed and the tail intensity of each comet image was measured using an image analysis software (Comet assay IV, Perceptive Instruments Ltd., Suffolk, UK).

Micronucleus (MN) test

The MN test for the testis was conducted according to the method of Tates et al. (1983) with a slight modification. Briefly, the testes excised from each animal were minced in HBSS Solution. The cell suspensions were incubated in 2 mg/ml collagenase solution (Wako Pure Chemicals Ind., Osaka, Japan) for 30 min at 37°C in a shaking water bath, filtered through a cell strainer, washed, and fixed in methanol. The cells were stained with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride), and the slide specimens were prepared with acridine orange coating (TOYOBO Co., Ltd., Tokyo, Japan). We observed 1,000 early spermatids per animal under a fluorescence microscope (BX50, Olympus Co.).

Measurement of GST activity and GSH content

The livers and testes obtained from the satellite groups were rinsed with PBS solution to remove any red blood cells. Total GSH concentrations were determined with the Glutathione Assay Kit (Cayman Chemical, Ann Arbor, MI, USA), and GST activity was measured using a Glutathione *S*-Transferase Assay Kit (Cayman Chemical). Sample preparation and measurement were conducted according to the instructions of the manufacturer.

Statistical analysis

Variance in data for body weights, food consumption, water consumption, values from morphometric assessment in the sciatic nerves, cerebellar molecular layer and testis, and data for the comet assay were checked for homogeneity by Bartlett's procedure. If the variance was homogeneous, the data were assessed by one-way analysis of variance. If not, the Kruskal–Wallis test was applied. When statistically significant differences were indicated, the Dunnett's multiple test was employed for comparisons between the 0 ppm and ACR-treated groups. Data for the MN tests, GST activity, and GSH content were analyzed by Student's or Welch's *t* test following a test for equal variance.

Results

In-life parameters and intake of ACR

Suppression of body weight gain was observed in the young groups at 100 and 200 ppm from week 2 (Fig. 1a).

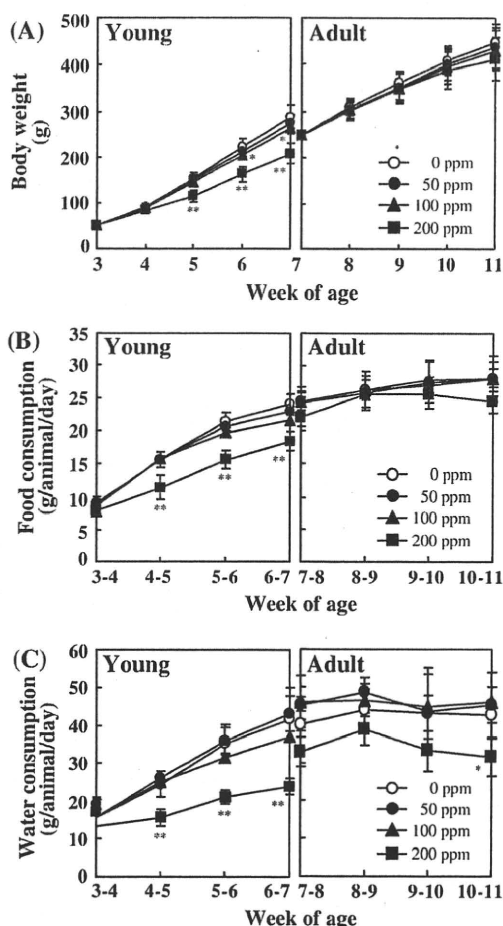

Fig. 1 Time course of change in body weights (a), food consumption (b), and water consumption (c) in young and adult rats given ACR in the drinking water for 4 weeks. Data are mean ± SD. *, ***P* < 0.05 and *P* < 0.01 vs. 0 ppm

In the adult groups, there were no intergroup differences in the body weight curves. Also, food consumption was suppressed only in the young group at 200 ppm (Fig. 1b). Water consumption was lowered at 200 ppm both in young and adult groups (Fig. 1c). Mean daily intakes of ACR are summarized in Table 1. Compared to adult groups, mean daily intake of ACR per kg body weight was higher in young groups at each dose.

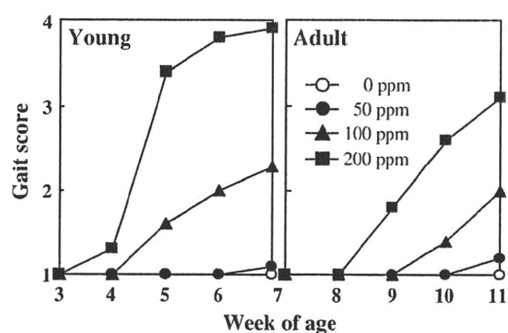

Fig. 2 Scores for gait abnormalities of young and adult rats given ACR in the drinking water for 4 weeks

Both in young and adult groups, apparent gait abnormalities were found at 100 and 200 ppm, and their severity advanced during the exposure in a dose-dependent manner (Fig. 2). Young groups showed earlier occurrence of gait abnormalities and faster progression of the symptoms than adult groups. At 200 ppm, slightly abnormal gait appeared in the young animals from week 1, and symptoms rapidly progressed so that the gait score reached 3.4 at week 2. Adult animals at 200 ppm exhibited mild gait abnormality from week 2, which progressed to score 3.1 at week 4.

Final body and organ weights are summarized in Table 2. In young groups, body weights were significantly depressed at 100 and 200 ppm. Alteration of the brain weight in young rats appeared to reflect body weight decrease. Decreases in absolute weights of the testis and epididymides observed in young and adult rats could have been linked with the histopathological changes described below.

Morphometric analysis

Data for histopathology and morphometry of lesions developing in the nervous system are shown in Table 3. Representative histopathological illustrations of the nervous systems of young and adult groups are summarized in Fig. 3 and Fig. 4, respectively. In both young and adult groups, central chromatolysis of ganglion cells in the trigeminal nerves was apparent from 100 ppm. The density of

Table 1 Mean daily intake of ACR in young and adult rats

Group	Acrylamide in the drinking water (ppm)				
		0	50	100	200
	No. of animals examined	10	10	10	10
Young	(mg/kg/day)	0 ± 0[a]	8.27 ± 0.32	15.73 ± 1.51	26.37 ± 3.51
Adult	(mg/kg/day)	0 ± 0	6.26 ± 1.10	12.63 ± 1.97	19.07 ± 3.46

[a] Mean ± SD