infants had a lower gestational age (38.16 ± 2.34 weeks) than MEHP-negative infants (39.35 ± 1.35 weeks). Intrauterine inflammation due to DEHP and/or MEHP exposure may be a risk factor for prematurity because intrauterine infection/inflammation is a major cause of premature labor. These studies suggest that DEHP may play a role in inducing the intrauterine inflammatory process.

Thelarche, premature breast development, is the growth of mammary tissue in young girls without other manifestations of puberty. Colon et al. (2000) analyzed serum samples from 41 Puerto Rican thelarche patients and 35 age matched controls. Significantly higher levels of DMP, DEP, DBP, DEHP, and MEHP were found in 28 (68%) samples from thelarche patients. This study suggested a possible association between PAEs and premature breast development. However, McKee et al. (2004) stated that the association between PAE exposure and thelarche seems highly unlikely for two reasons. First, the reported exposure levels of PAEs may have reflected contamination since they were very high when compared to recent exposure information. Second, toxicological evidence from the laboratory studies described below do not support any influence on female sexual development.

DEHP exposure at 2000 mg/kg/day for 1–12 days in mature SD rats resulted in decreased serum estradiol levels, prolonged estrous cycles and no ovulation (Davis et al., 1994). A two generation reproductive study in SD rats revealed that oral doses of 500 mg/kg/day BBP caused atrophy of the ovary in one female and significant decreases in absolute and relative ovary weights. However oral doses of up to 500 mg/kg/day BBP did not affect estrous cycles in SD rats (Nagao et al., 2000). Similarly, when DEHP was administered to rats over two generations at up to 9000 ppm (about 900 mg/kg/day) in the diet, there were no effects on the pattern and duration of the estrous cycle in F0 female rats (Schilling et al., 1999). Histological changes in female reproductive organs were also not observed after exposure to di-*n*-propyl phthalate, DBP, di-*n*-pentyl phthalate, DHP, or DEHP (Heindel et al., 1989; Lamb et al., 1987). Although some PAEs have been reported to be weakly estrogenic in estrogen-responsive human breast cancer cells (Jobling et al., 1995; Sonnenschein et al., 1995; Soto et al., 1995; Zacharewski et al., 1998) and/or in a recombinant yeast screen (Coldham et al., 1997; Harris et al., 1997), no PAEs showed any estrogenic response upon in vivo uterotrophic or vaginal cornification assay (Zacharewski et al., 1998). Thus, there is no evidence that PAEs influence the timing of female sexual development in laboratory studies.

3.3. Studies in human infants

Anogenital distance (AGD) is a developmental landmark for the differentiation of the external genitalia and is commonly used as a hormonally sensitive parameter of sex differentiation in rodents. AGD in male rats is normally about twice that in females, and a similar sex difference is observed in humans (Salazar-Martinez et al., 2004). Many studies in male rodents reported a reduction of AGD after prenatal exposure to PAEs (Table 7). Chemicals that adversely affect human sex differentiation (Schardein, 2000) also produce predictable alterations of this process in rodents (Gray et al., 1994). In a Hershberger assay, significant decreases in seminal vesicles, ventral prostate, levator ani/bulbocavernosus muscles weights were observed in animals treated with DEHP, DBP, DINP, di-isodecyl phthalate or MEHP, which suggest that some phthalates possess anti-androgenic activity (Lee and Koo, 2007). Swan et al. (2005) presented the first study of AGD and other genital measurements in relation to PAE exposure in a human population. AGD data were obtained for 134 boys of 2–36 months of age. Mother's urine during pregnancy was assayed for phthalate metabolites. Urinary concentrations of four phthalate metabolites, MEP, MBP, MBzP, and mono-isobutyl phthalate (MiBP), were negatively related to the anogenital index (AGI) which is a weight-normalized index of AGD [AGD/weight (mm/kg)].

In rats, undescended testes were observed in male pups after maternal dosing of BBP, MBzP, DEHP, DBP, or MBP (Table 7). Main et al. (2006) investigated whether phthalate monoesters in human breast milk had any relation to cryptorchidism in newborn boys (1–3 months of

Table 7
Decreased AGD and undescended testes observed in experimental animals

Compounds	Animals	Days of administration	Route	Dose (mg/kg/day)	Decreased male AGD	Undescended testes	Reference
BBP	Wistar rat	GDs 15–17	Gavage	500	+	+	Ema and Miyawaki (2002)
				1000	+	+	
MBzP	Wistar rat	GDs 15–17	Gavage	250	+	+	Ema et al. (2003)
				375	+	+	
DEHP	SD rat	GD 2-PND 21	Gavage	750	+		Moore et al. (2001)
				1500	+	+	
DBP	Wistar rat	GDs 11–21	Diet	555	+	+	Ema et al. (1998)
				661	+	+	
DBP	Wistar rat	GDs 15–17	Gavage	500	+	+	Ema et al. (2000)
				1500	+	+	
MBP	Wistar rat	GDs 15–17	Gavage	250	+	+	Ema and Miyawaki (2001)
				500	+	+	

age). The median levels of MMP, MEP, MBP, MBzP, MEHP, and MINP in breast milk were 0.10, 0.95, 9.6, 1.2, 11, and 95 μg/L, respectively. No association was found between phthalate monoester levels and cryptorchidism. However, there were positive correlations for MEP and MBP with sex hormone-binding globulin, MMP, MEP, and MBP with the ratio of LH/free testosterone, and MINP with LH. MBP was negatively correlated with free testosterone. These mother–son cohort studies provided evidence that testicular and genital development may also be vulnerable to perinatal exposure to PAEs.

Although these two studies of human infants indicate possible associations between PAE exposure and the development of the human reproductive system, two follow-up studies of adolescents exposed to DEHP from medical devices as neonates showed no significant adverse effects on their maturity or sexual activity. A comparison of very low birth weight infants who had undergone neonatal intensive care and infants with normal birth weights showed that there were no differences in the rates of sexual intercourse, pregnancy, or live births when the infants became young adults (Hack et al., 2002). Another study indicated that adolescents exposed to DEHP as neonates showed no significant adverse effects on physical growth and pubertal maturity. Thirteen male and 6 female subjects of 14–16 years of age who had undergone extracorporeal membrane oxygenation as neonates had a complete physical examination to evaluate the long-term toxicity of DEHP in infants. Thyroid, liver, renal, and male and female gonadal functions tested were within normal ranges for the given age and sex distribution (Rais-Bahrami et al., 2004).

4. Overall conclusions

In conclusion, exposure data in human populations indicate that the current methodology of estimation of PAEs is inconsistent. It is important to obtain improved data on human PAE exposure and a better understanding of the toxicokinetics of PAEs in each subpopulation. Oxidized metabolites of DEHP and DINP were recently recognized as the major urinary metabolites in humans (Barr et al., 2003; Koch and Angerer, 2007; Koch et al., 2004a, 2005b). These findings could be useful to establish new hypotheses for laboratory studies. Hauser et al. (2007) found that oxidative metabolites of DEHP had a negative association with sperm DNA damage, suggesting that the oxidation of MEHP to 5OH-MEHP and 5oxo-MEHP is protective against sperm DNA damage. However, in an in vitro study, 5OH-MEHP and 5oxo-MEHP, but not DEHP or MEHP, were antiandrogenic (Stroheker et al., 2005). The relevance of this in vitro study to findings in human populations is not clear. Therefore, further studies are required to facilitate accurate risk assessments for human health.

Studies of health effects of PAEs in humans have remained controversial due to limitations of the study designs. Some findings in human populations are consistent with animal data suggesting that PAEs and their metabolites produce toxic effects in the reproductive system. However, it is not yet possible to conclude whether phthalate exposure is harmful for human reproduction. Studies in humans have to be interpreted cautiously because they are conducted in a limited number of subjects. Spot samples only reflect recent phthalate exposure due to the short half-life and it has not yet been confirmed whether point estimates are representative of patterns of long exposure, although reproducibility was found for urinary phthalate monoester levels over two consecutive days (Hoppin et al., 2002). The timing of exposure is a critical factor for decreased AGD in animal studies (Ema and Miyawaki, 2001); however, the stage of fetal development was unknown at the time of urine sampling in the study of Swan et al. (2005). Further studies need to be conducted to confirm these results in human populations and identify the potential mechanisms of interaction.

The studies in human populations reviewed in this paper are useful for showing the strength of associations. Evidence from human studies is preferred for risk assessment as long as it is obtained humanely. It is sometimes claimed that the use of animal data for estimating human risk dose not provide strong scientific support. However, because it is difficult to find alternative methods to test the direct toxic effects of chemicals, continuance of studies in animals is required for risk assessment of chemicals including PAEs.

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Genes and Environment, Vol. 28, No. 4 pp. 141–152 (2006)

Commentary

Globally Harmonized System on Hazard Classification and Labeling of Chemicals and Other Existing Classification Systems for Germ Cell Mutagens

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(Received August 8, 2006; Revised August 26, 2006; Accepted August 28, 2006)

The Globally Harmonized System (GHS) on hazard classification and labeling of chemicals will be implemented globally by 2008. The GHS includes (a) harmonized criteria for classifying chemicals and chemical mixtures according to their health, environmental and physical hazards, and (b) harmonized hazard communication elements, including requirements for labeling and safety data sheets. Germ cell mutagenicity is included in the GHS health hazard classes in addition to carcinogenicity. This means increased significance for then results of genetic toxicology testing for the classification of chemicals. GHS requires the classification of chemicals if they are germ cell mutagens (categories 1A, 1B and 2) or not. Several classification systems for germ cell mutagens have been proposed in the EU, Germany, US, Canada, in advance of the adoption of the GHS. In this paper, these classification systems including GHS are introduced and summarized to provide the basis of the hazard classification of germ cell mutagens. Though the objectives, target audiences and criteria of these classification systems are different, the GHS will become standard for hazard classification. Hazard classification is a significant first step in risk communication. Further development of risk evaluation criteria and communication on germ cell mutagens is expected.

Key words: GHS, hazard classification, germ cell mutagenicity, germ cell mutagens

Introduction

The Globally Harmonized System (GHS) of classification and labeling of chemicals is a single, globally harmonized system to address classification of chemicals, labelling, and safety data sheets, which has been developed by the United Nations (UN). The GHS document has been prepared and published by the secretariat of the United Nations Economic Commission for Europe (1,2). The GHS covers all hazardous chemicals except for pharmaceuticals, food additives, cosmetics, and pesticide residues in food in terms of labeling at the point of intentional intake. The GHS is based on currently available data and thus compliance with these criteria will not require retesting of chemicals for which acceptable test data already exists.

The goal of the GHS is to identify the intrinsic hazards found in chemicals and chemical mixtures and to convey the information about these hazards to the target audiences including consumers, workers, transport workers, and emergency responders. The World Summit on Sustainable Development in Johannesburg on 4th September, 2002 encouraged countries to implement the GHS as soon as possible with a view to having the system fully operational by 2008. In Japan, an interministerial committee was organized in 2001 to share information about the GHS among ministries and to play a pivotal role in the UN-Subcommittee. Seven government offices, *i.e.*, Ministry of Health, Labour and Welfare (MHLW), Ministry of Economy, Trade and Industry (METI), Ministry of the Environment (MOE), Ministry of Internal Affairs and Communications (MIC), Ministry of Agriculture, Forestry and Fisheries (MAFF), Ministries of Land Infrastructure and Transport (MLIT) and Ministry of Foreign Affairs (MOFA), and experts from national laboratories and industries participated in the committee. The committee's activities include: (i) translation of the GHS into Japanese (3); (ii) information sharing among ministries with respect to the relevant domestic laws; (iii) classification of chemicals under each relevant domestic law (4); and (iv) deliberation on the agenda items and documents of the UN Sub-Committee meetings and decision-making about the Japanese position.

The GHS includes the following two elements: harmonized criteria for classifying substances and mixtures according to their health (10 hazard classes),

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environmental (1 hazard class) and physical hazards (16 hazard classes); and harmonized hazard communication elements, including requirements for labeling and safety data sheets. The GHS requests classification of the following 10 hazard classes in health hazard, *i.e.*, (i) acute toxicity, (ii) skin corrosion/irritation, (iii) serious eye damage/eye irritation, (iv) respiratory or skin sensitization, (v) germ cell mutagenicity, (vi) carcinogenicity, (vii) reproductive toxicity, (viii) specific target organ systemic toxicity—single exposure, (ix) specific target organ systemic toxicity—repeated exposure, and (x) aspiration hazard.

It is striking that not mutagenicity per se but germ cell mutagenicity specifically is included in GHS health hazard class in addition to carcinogenicity. The GHS focuses on heritable effects by mutagens. Several classification systems of mutagens or germ cell mutagens have been proposed from European Union (EU), Germany, United States of America (US), Canada, etc. The criteria for germ cell mutagens of GHS and other systems are reviewed and summarized to aid understanding of the control of chemicals by these regulations.

Classification Systems for Germ Cell Mutagens

Classification systems for mutagens or germ cell mutagens in GHS, EU, Germany, USA, Canada, and Japan are described below.

GHS: In the GHS (2), the term "mutation" applies both to heritable genetic changes that may be manifested at the phenotypic level and to the underlying DNA modifications when known (including, for example, specific base pair changes and chromosomal translocations). The term "mutagenic" and "mutagen" will be used for chemicals giving rise to an increased occurrence of mutations in populations of cells and/or organisms. The more general terms "genotoxic" and "genotoxicity" apply to chemicals or processes which alter the structure, information content, or segregation of DNA, including those which cause DNA damage by interfering with normal replication processes, or which in a non-physiological manner (temporarily) alter its replication.

In order to achieve classification, GHS states that 'Test results are considered from experiments determining mutagenic and/or genotoxic effects in germ and/or somatic cells of exposed animals. The system is hazard based, classifying chemicals on the basis of their intrinsic ability to induce mutations in germ cells. The scheme is, therefore, not meant for the (quantitative) risk assessment of chemical substances. Classification for heritable effects in human germ cells is made on the basis of well conducted, sufficiently validated tests, preferably as described in OECD Test Guidelines. Evaluation of the test results should be done using expert judgment and all the available evidence should be weighed for classification. The classification of individual substances should be based on the total weight of evidence available, using expert judgment. In those instances where a single well-conducted test is used for classification, it should provide clear and unambiguously positive results. If new, well validated, tests arise these may also be used in the total weight of evidence to be considered. The relevance of the route of exposure used in the study of the chemical compared to the route of human exposure should also be taken into account.'

The criteria for classification of germ cell mutagens places chemicals in one of three categories, category 1 being used for chemicals known to induce heritable mutations (category 1A) or known to be regarded as if they induce heritable mutations in germ cells of humans (category 1B); category 2 for chemicals which cause concern for humans owing to the possibility that they may induce heritable mutations in the germ cells of humans (2). The criterion for category 1A is positive evidence from human epidemiological studies. The criteria for category 1B are as follows: (i) positive result(s) from *in vivo* heritable germ cell mutagenicity tests in mammals; or (ii) positive result(s) from *in vivo* somatic cell mutagenicity tests in mammals, in combination with some evidence that the substance has the potential to cause mutations to germ cells. This supporting evidence may, for example, be derived from mutagenicity/genotoxic tests in germ cells *in vivo*, or by demonstrating the ability of the substance or its metabolite(s) to interact with the genetic material of germ cells; or (iii) positive results from tests showing mutagenic effects in the germ cells of humans, without demonstration of transmission to progeny; for example, an increase in the frequency of aneuploidy in the sperm cells of exposed people. The criteria for category 2 are positive evidence obtained from experiments in mammals and/or in some cases from *in vitro* experiments, obtained from: (i) somatic cell mutagenicity tests *in vivo*, in mammals; or (ii) other *in vivo* somatic cell genotoxicity tests which are supported by positive results from *in vitro* mutagenicity assays. In addition, following criteria are included as *Note*: Chemicals which are positive in *in vitro* mammalian mutagenicity assays, and which also show chemical structure activity relationship to known germ cell mutagens, should be considered for classification as Category 2 mutagens.

If there are not enough data for the evaluation of mutagenicity of the chemical, it regards as "classification not possible" (Fig. 1). If there is no concern of induction of heritable mutations in the germ cells of humans or insufficient evidence for inclusion in category 1 or 2, the chemicals are regarded as "not classified". Hazard categories and their criteria for germ cell mutagens in GHS are summarized in Table 1. For classification of chemical mixtures, the mixture will be

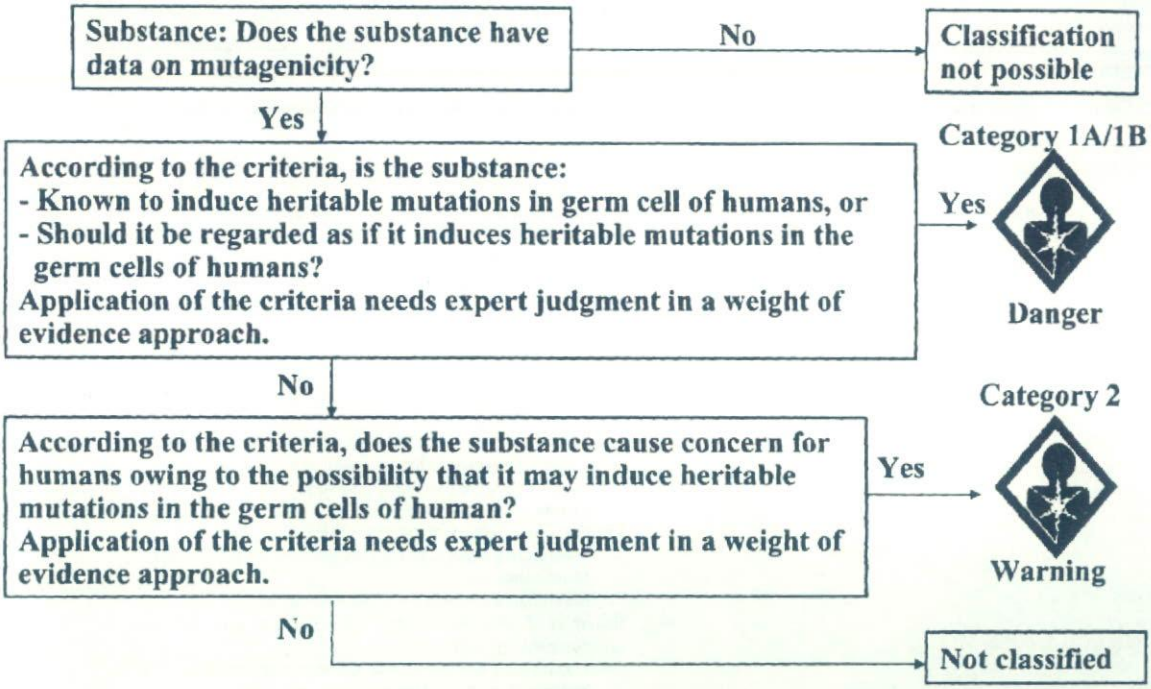

Fig. 1. Decision logic for the classification of germ cell mutagens in GHS (2)

classified as a mutagen when at least one ingredient has been classified as a category 1 or category 2 mutagen and is present at or above the cut-off value/concentration limits below for category 1 ($\geq 0.1\%$) or category 2 ($\geq 1.0\%$), respectively.

European Union (EU): The criteria for classification of mutagens in EU are described in Commission directive (5, 6). There are three categories: substances known to be mutagenic to human (category 1) for which there is sufficient evidence to establish a causal association between human exposure to the chemical and heritable genetic damage; substances which should be regarded as if they were mutagenic to human (category 2) for which there is sufficient evidence to provide a strong presumption that human exposure to the chemical may result in the development of heritable genetic damage, generally on the basis of appropriate animal studies and other relevant information; substances which cause concern for humans owing to possible mutagenic effect (category 3) for which there is evidence from appropriate mutagenicity studies, but it is insufficient to place the substance in category 2. EU criteria for classification of chemicals are summarized in Table 2.

This system is primarily based on intrinsic hazard, despite the statement in the Annex (7) that 'the object of classification is to identify all the physicochemical, toxicological and ecotoxicological properties of substances and preparations which may constitute a risk during normal handling or use' (4).

Germany: Maximale Arbeitsplatz-Konzentration (MAK) Commission in Germany proposed 5 categories for classification of germ cell mutagens at the workplace (8, 9). These are germ cell mutagens which have been shown to increase the mutant frequency in the progeny of exposed humans (category 1); germ cell mutagens which have been shown to increase the mutant frequency in the progeny of exposed mammals (category 2); chemicals which have been shown to induce genetic damage in germ cells of humans and/or animals, or which produce mutagenic effects in somatic cells of mammals *in vivo* and the chemicals have been shown to reach the germ cells in an active form (category 3A); chemicals which are suspected of being germ cell mutagens because of their genotoxic effects in mammalian somatic cells *in vivo*; in exceptional cases, chemicals for which there are no *in vivo* data but which are clearly mutagenic *in vitro* and structurally related to known *in vivo* mutagens (category 3B); and germ cell mutagens, the potency of which is considered to be so low that, provided the MAK value (Maximum Concentration at the Workplace) is observed, their contribution to genetic risk for man is expected not to be significant (category 5). Category 4 is not applicable in germ cell mutagenicity because this classification system has been

Table 1. GHS classification on germ cell mutagens (1,2)

Category	Classification	Criteria
Category 1A	Chemicals known to induce heritable mutations in germ cells of humans	Positive evidence from human epidemiological studies.
Category 1B	Chemicals which should be regarded as if they induce heritable mutations in the germ cells of humans	—Positive result(s) from *in vivo* heritable germ cell mutagenicity tests in mammals; or —Positive result(s) from *in vivo* somatic cell mutagenicity tests in mammals, in combination with some evidence that the substance has potential to cause mutations to germ cells. This supporting evidence may, for example, be derived from mutagenicity/genotoxic tests in germ cells *in vivo*, or by demonstrating the ability of the substance or its metabolite(s) to interact with the genetic material of germ cells; or —Positive results from tests showing mutagenic effects in the germ cells of humans, without demonstration of transmission to progeny; for example, an increase in the frequency of aneuploidy in sperm cells of exposed people. Examples of *in vivo* heritable germ cell mutagenicity tests are: — Rodent dominant lethal mutation test — Mouse heritable translocation assay — Mouse specific locus test Examples of *in vivo* somatic cell mutagenicity test are: — Mammalian bone marrow chromosome aberration test — Mouse spot test — Mammalian erythrocyte micronucleus test Examples of mutagenicity/genotoxicity tests in germ cells are: (a) Mutagenicity tests: — Mammalian spermatogonial chromosome aberration test — Spermatid micronucleus assay (b) Genotoxicity tests: — Sister chromatid exchange analysis in spermatogonia — Unscheduled DNA synthesis test (UDS) in testicular cells
Category 2	Chemicals which cause concern for humans owing to the possibility that they may induce heritable mutations in the germ cells of humans	—Positive evidence obtained from experiments in mammals and/or in some cases from *in vitro* experiments, obtained from: —Somatic cell mutagenicity tests *in vivo*, in mammals; or —Other *in vivo* somatic cell genotoxicity tests which are supported by positive results from *in vitro* mutagenicity assays. Examples of genotoxicity tests in somatic cells are: — Liver UDS *in vivo* — Mammalian bone marrow sister chromatid exchanges Examples of *in vitro* mutagenicity tests are: — *In vitro* mammalian chromosome aberration test — *In vitro* mammalian cell gene mutation test — Bacterial reverse mutation tests Note: Chemicals which are positive in *in vitro* mammalian mutagenicity assays, and which also show chemical structure activity relationship to known germ cell mutagens, should be considered for classification as Category 2 mutagens.

established in analogy to the categories for carcinogenic chemicals. Category 4 carcinogenic substances are those with non-genotoxic mechanisms of action. By definition, germ cell mutagens are genotoxic. MAK categories and criteria for classification of germ cell mutagens are summarized in Table 3.

The MAK Commission describes germ cell mutagenicity as follows (9): 'Germ cell mutagens produce heritable gene mutations, and heritable structural and numerical chromosome aberrations in germ cells. The consequences of germ cell mutations in subsequent generations include genetically determined phenotypic alterations without signs of illness, reduction in fertility, embryonic or perinatal death, more or less severe congenital malformations, and genetic diseases with various degrees of health impairment. The term "germ cell mutagenicity" refers specifically to mutagenicity in male and female germ cells and is distinguished from mutagenicity in somatic cells, which can initiate cancer. Epidemiological studies, however, have been unable to

Table 2. EU criteria for classification of chemicals as mutagenic (6,7)

Category	Classification	Criteria
Category 1	Substances known to be mutagenic to human	Positive evidence from human mutation epidemiology studies will be needed. Examples of such substances are not known to date.
Category 2	Substances which should be regarded as if they are mutagenic to human	Positive results from assays showing (a) mutagenic effects, or (b) other cellular interactions relevant to mutagenicity, in germ cells of mammals *in vivo*, or (c) mutagenic effects in somatic cells of mammals *in vivo* in combination with clear evidence that the substance or a relevant metabolite reaches the germ cells. With respect to placement in category 2, at present the following methods are appropriate:2(a) *In vivo* germ cell mutagenicity assays: — specific locus mutation test, — heritable translocation test, — dominant lethal mutation test. These assay actually demonstrate the appearance of affected progeny or a defect in the developing embryo. 2(b) *In vivo* assays showing relevant interaction with germ cells (usually DNA): — assays for chromosomal abnormalities, as detected by cytogenetic analysis, including aneuploidy, caused by malsegregation of chromosomes, — test for sister chromatid exchanges (SCEs), — test for unscheduled DNA synthesis (UDS), — assay of (covalent) binding of mutagen to germ cell DNA, — assaying other kinds of DNA damage. These assays provide evidence of a more or less indirect nature. Positive results in these assays would normally be supported by positive results from *in vivo* somatic cell mutagenicity assays, in mammals or in man. 2(c) *In vivo* assays showing mutagenic effects in somatic cells of mammals, in combination with toxicokinetic methods, or other methodologies capable of demonstrating that the compound or a relevant metabolite reaches the germ cells. For 2(b) and 2(c), positive results from host-mediated assays or the demonstration of unequivocal effects in *in vitro* assays can be considered as supporting evidence.
Category 3	Substances which cause concern for human owing to possible mutagenic effects.	There is evidence from appropriate mutagenicity studies, but this is insufficient to place the substance in category 2. Assays showing (a) mutagenic effects or (b) other cellular interaction relevant to mutagenicity, in somatic cells in mammals *in vivo*. The latter would be supported by positive results from *in vitro* mutagenicity assays. For effects in somatic cells *in vivo* at present the following methods are appropriate: 3(a) *In vivo* somatic cell mutagenicity assays: — bone marrow micronucleus test or metaphase analysis, — metaphase analysis of peripheral lymphocytes, — mouse coat color spot test. 3(b) *In vivo* somatic cell DNA interaction assays: — test for SCEs in somatic cells, — test for UDS in somatic cells, — assay for the (covalent) binding of mutagen to somatic cell DNA, — assay for DNA damage, e.g, by alkaline elution, in somatic cells. Substances showing positive results only in one or more *in vitro* mutagenicity assays should normally not be classified. Their further investigation using *in vivo* assays, however, is strongly indicated. In exceptional cases, e.g., for a substance showing pronounced responses in several *in vitro* assays, for which no relevant *in vivo* data are available, and which shows resemblance to known mutagens/carcinogens, classification in category 3 could be considered.

provide any evidence as yet that exposure to chemicals or to radiation results in hereditary diseases in man. Although structural changes have been demonstrated in the chromosomes of the germ cells of men exposed to radiation, even this finding can only provide indirect evidence that such exposures could lead to hereditary disorders in the offspring. The proof that an increased frequency of hereditary diseases is related to a particular exposure would be associated with great methodological difficulties. In the human population there are a large number of hereditary diseases of unknown origin with frequencies that differ widely in different subpopulations. Since mutational events occur largely randomly in the genome, it is not to be expected that one particular substance would induce one characteristic genetic disease. Therefore, it is most unlikely that proof of a

Table 3. Categories for classification of germ cell mutagens by MAK commission (8,9)

Category	Classification	Criteria
Category 1	Substances shown to increase the mutant frequency in the progeny of exposed humans	In the section 'Epidemiological methods and their limitations' it is explained why epidemiological studies to date have not been able to prove that the exposure of a particular human population to a particular substance has resulted in an increase in the incidence of inherited mutations. This is true both for ionizing radiation and chemical mutagens. Even if epidemiological methods are improved further, it is unlikely that such proof will be available in the foreseeable future. Category 1 will therefore probably remain without any entries.
Category 2	Substances shown to increase the mutant frequency in the progeny of exposed mammals	Classified as category 2 are substances that increase the incidence of genetically modified live progeny in animal studies, for example in the specific locus test or in the test for heritable translocations. Likewise, substances that should be classified as category 2 are those that increase the incidence of embryos that die in utero, for example in the dominant lethal test.
Category 3A	Substances shown to induce genetic damage in germ cells of humans or animals, or which produce mutagenic effects in somatic cells of mammals *in vivo* and shown to reach the germ cells in an active form	The methods include tests for genotoxicity in germ cells of experimental animals, such as tests for induction of structural chromosomal changes in spermatogonia or spermatocytes, for sister chromatid exchange in spermatogonia, for micronuclei in round spermatids, for numerical chromosome changes in secondary spermatocytes or in spermatozoa, for DNA single strand breaks and for repair synthesis or for covalent binding to the DNA. Also relevant are the observations obtained from exposed human populations which provide evidence for structural or numerical chromosome changes in spermatozoa of exposed persons. The development of new methods, especially molecular genetic methods for the detection of gene mutations in germ cells is to be expected. Substances that yield positive results in tests with germ cells are classified as category 3A. Also taken into account are clearly positive results from *in vivo* tests for mutagenicity in somatic cells, for example chromosomal aberrations or micronuclei in bone marrow cells, somatic mutations in the mammalian spot test or transgenic animals, provided that it has been demonstrated that the active substance or an active metabolite reaches the germ cells after relevant exposure of the experimental animals. Such substances are also suspected of being mutagenic in germ cells. Therefore they are classified as category 3A.
Category 3B	Substances suspected of germ cell mutagens because of their genotoxic effects in mammalian somatic cells *in vivo*; in exceptional cases, substances without *in vivo* data but with clearly mutagenic *in vitro* and structurally related to known *in vivo* mutagens	If the available data are not sufficient for classification in category 3A but the substance is clearly genotoxic in somatic cells of exposed animals or humans, the substance is also suspected of being mutagenic in germ cells. Substances that have yielded positive results in one or several *in vitro* mutagenicity tests generally not classified as category 3B. An exception is made for substances for which there are no relevant *in vivo* data but which are clearly genotoxic *in vitro* and also structurally related to substances known to be genotoxic *in vivo*. Such substances raise concern and are classified as category 3B.
Category 4	Not applicable	Category 4 carcinogenic substances are those with non-genotoxic mode of action. By definition, germ cell mutagens are genotoxic. Therefore, a category 4 for germ cell mutagens cannot exist. Depending on future research results, a category 4 could be defined at a later time for genotoxic substances with targets other than DNA (*i.e.*, pure aneugens).
Category 5	Substances considered the potency is considered so low, their contribution to genetic risk for man is expected not to be significant	Substances classified as category 5 are not expected to contribute significantly to the genetic risk for humans provided the MAK value is observed. For classification in this category, information on the spectrum of effects and their dose-dependence, and toxicokinetic data for species comparison are required. Biochemical and biological end-points can be used to characterize the contribution to genetic risk. The contribution to genetic risk is considered not to be significant after exposure at the workplace if the internal exposure level of the substance or its biomarkers is in the range of the background levels in a not specifically exposed reference population: @ Under workplace conditions the levels of biochemical effect markers such as DNA and protein adducts are not significantly increased above the background levels. @ Physiological-toxicokinetic model calculations based on animal data do not reveal a significant genetic risk for humans.

causal relationship between exposure to a chemical and occurrence of heritable diseases will become available in the foreseeable future. In this situation, for the identification of germ cell mutagens the results of animal experiments must be given particular attention. The mutagenic effect of chemicals on the germ cells of exposed parent animals can be demonstrated by observing an increased mutant frequency among the progeny. In addition, the demonstration of genotoxic effects of a substance in germ cells or somatic cells provides evidence of a potential hazard for subsequent generations.'

United States of America (US): *US EPA*: A classification using the following three categories of germ cell mutagens was proposed by the US Environmental Protection Agency (EPA) in 1984 for the evaluation of chemicals with respect to their ability to induce mutations in mammalian germ cells (10,11). Category I is based on sufficient evidence obtained from at least one *in vivo* mammalian germ cell mutation test or from at least two *in vivo* somatic cell mutation tests (point mutation and/or chromosomal aberrations), plus sufficient *in vivo* evidence that the chemical interacts with mammalian germ cells. Category II is based on suggestive evidence provided from positive results of *in vivo* somatic cell mutation tests plus evidence for interaction of the chemical with mammalian germ cells, but the evidence is insufficient to place the chemical in Category I. Category III is based on limited evidence of *in vivo* mutagenic activity or interaction of the chemical with mammalian germ cell DNA or other chromatin constituents. In 1986, guidelines for a "weight-of-evidence" approach to human germ cell mutagenicity were established, leading to eight categories with a decreasing order of strength of evidence (10). In addition, a "non-mutagen" category and a category for substances with inadequate evidence were described. The eight categories of evidence are as follows: (i) positive data derived from human germ cell mutagenicity studies; (ii) valid positive results from studies on heritable mutational events (of any kind) in mammalian germ cells; (iii) valid positive results from mammalian germ cell chromosome aberration studies that do not involve transmission from one generation to the next; (iv) sufficient evidence for a chemical's interaction with mammalian germ cells, together with valid positive mutagenicity test results from two assay systems, at least one of which is mammalian (*in vivo* or *in vitro*). The positive results may be both for gene mutation and chromosomal aberrations in mammalian systems; (v) suggestive evidence for a chemical's interaction with mammalian germ cells, together with valid positive mutagenicity evidence from two assay systems as described above under #iv. Alternatively, positive mutagenicity evidence of less strength than defined under #iv, when combined with sufficient evidence for a chemical's interaction with mammalian germ cells; (vi) positive mutagenicity test results of less strength than defined under #iv, combined with suggestive evidence for a chemical's interaction with mammalian germ cells; (vii) although definitive proof of non-mutagenicity is not possible, a chemical could be operationally classified as a non-mutagen for human germ cells if it gives valid negative test results for all endpoints of concern; and (viii) inadequate evidence bearing on either mutagenicity or chemical interaction with mammalian germ cells.

This system is for the classification for transmissible germ cell genetic risk. The Guideline (10) describes 'Evidence that an agent induces heritable mutations in human beings could be derived from epidemiologic data indicating a strong association between chemical exposure and heritable effects. It is difficult to obtain such data because any specific mutation is a rare event, and only a small fraction of the estimated thousands of human genes and conditions are currently useful as markers in estimating mutation rates. Human genetic variability, small numbers of offspring per individual and long generation times further complicate such studies. In addition, only disorders caused by dominant mutations, some sex-linked recessive mutations, and certain chromosome aberrations can be detected in the first generation after their occurrence. Conditions caused by autosomal recessive disorders (which appear to occur more frequently than dominant disorders) or by polygenic traits may go unrecognized for many generations. Therefore, in the absence of human epidemiological data, it is appropriate to rely on data from experimental animal systems as long as the limitations of using surrogate and model systems are clearly stated. Despite species differences in metabolism, DNA repair, and other physiological processes affecting chemical mutagenesis, the virtual universality of DNA as the genetic material and of the genetic code provides a rationale for using various nonhuman test systems to predict the intrinsic mutagenicity of test chemicals. Additional support for the use of nonhuman systems is provided by the observation that chemicals causing genetic effects in one species or test system frequently cause similar effects in other species or systems. Evidence also exists that chemicals can induce genetic damage in somatic cells of exposed humans. Furthermore, a wide variety of different types of mutations have been observed in humans, including numerical chromosome aberrations, translocations, base-pair substitutions, and frameshift mutations. Although the cause of these mutations is uncertain, it is clear from these observations that human germ-cell DNA is subject to the same types of mutational events that are observed in other species and test systems.'

***Recent US EPA proposals*:** Dearfield *et al.* from

Table 4. Proposed mutagenicity classification categories by US EPA researchers (12)

Classification	Criteria in Somatic cells	Criteria in Germ cells
Human mutagen	Positive in human somatic cell mutagenicity studies as a result of human *in vivo* exposure. May be human carcinogens; unless the risk characterization suggest not as likely. This can include cytogenetic endpoints in tissues (such as lymphocytes) from exposed persons.	Positive in human *in vivo* germ cell mutagenicity studies. Human germ cell mutagens. This is based on positive *in vivo* findings from appropriate germ cell targets in exposed humans. It is recognized that a human germ cell mutagen is not currently identified.
Probable human mutagen	Clear evidence for genotoxic activity *in vivo* mammalian test(s), usually supported by *in vitro* test(s). Usually animal carcinogens and may be human carcinogens. Classification at this level usually means that some *in vivo* testing has been performed as follow-up to positive results from *in vitro* testing. Therefore, there is usually some supporting positive evidence from *in vitro* testing.	Sufficient evidence of interaction with mammalian germ cells with clear evidence for genotoxic activity. Includes valid positive results from studies on heritable mutational events in mammalian germ cells; or, valid positive results from mammalian germ cell chromosomal aberration studies that do not include an intergeneration test. Putative human germ cell mutagens if they reach target cells. Alternatively, it should produce positive results for DNA strand breaks (e.g. comet test), UDS, SCE and/or chromosome aberrations in germinal cells.
Possible human mutagen	Some evidence for genotoxic activity. May be carcinogenic through genotoxic mechanisms; possibly in humans. Confirmed positive results may be seen in the *in vitro* test systems without supporting evidence from the *in vivo* assays. Agents falling into this category are considered to have intrinsic mutagenic potential which is not detected *in vivo*.	Suggestive evidence of interaction with mammalian germ cells with some evidence for genotoxic activity. May be putative human germ cell mutagens if they reach target cells. For a test agent to be considered to present suggestive evidence of germ cell interaction, data are needed to demonstrate that: (i) the test agent shows some evidence of somatic cell mutagenicity and/or genotoxicity; (ii) the test agent reaches the gonads (e.g. data from pharmacokinetic/tissue distribution studies of the test agent and/or metabolites); (iii) the test agent interacts with germinal cells; these data come from subchronic or chronic toxicity tests showing gonadal pathology (e.g. sperm abnormalities); and (iv) the test agent causes adverse effects on reproductive parameters (e.g. decreased fertility, increased dead implants, reduced litter sizes).
Equivocal evidence	Results from acceptable tests that cannot be convincingly called negative or positive.	
Negative evidence	Negative results in acceptable tests.	

the US EPA proposed 6 categories of mutagenicity classification that are divided to 3 categories each for somatic cells and germ cells (12). This includes (i) human somatic cell mutagens in which positive data in human somatic cells are derived from studies with exposed humans that gives positive data in human; (ii) human germ cell mutagen in which positive data from human *in vivo* germ cell studies are obtained; (iii) probable human somatic cell mutagen in which clear evidence for genotoxic activity from positive *in vivo* mammalian test(s); (iv) probable human germ cell mutagen in which sufficient evidence of interaction with mammalian germ cells with clear evidence for genotoxic activity; (v) possible human somatic cell mutagen in which confirmed positive results may be seen in the *in vitro* test systems without supporting evidence from the *in vivo* assays; and (vi) possible human germ cell mutagen in which suggestive evidence of interaction with mammalian germ cells is seen. The mutagenicity classification categories proposed by Dearfield *et al.* (12) are summarized in Table 4.

The mutagenicity/genotoxicity data organized into "clear" and "some" evidence for mutagenicity and into "suggestive" and "sufficient" evidence germ cell interaction. The classification of mutagenicity results falls into the broad categories of inadequate, negative, equivocal, and positive data. With respect to the effects of mutagens, Dearfield *et al.* stated that 'In addition to cancer, adverse health effects from somatic cell mutations and/or germ cell mutations include sickle cell anemia, cardiovascular disease, reproductive/developmental effects, and neurobehavioral effects among many specific and general endpoints, as well as having impact on the aging process.' (12).

Canada: *Health Canada*: Health Canada proposed 6 categories of classification of chemicals with respect to

their mutagenic potential for germ cells in the Canadian Environmental Protection Act (CEPA), Human Health Risk Assessment for Priority Substances (13). Four categories have several subgroups based on the degree of evidence (Table 5). These classifications are as follows: human germ cell mutagen (group I), for which data from adequate epidemiological studies indicate that there is a causal relationship between exposure of humans to a chemical and an increased incidence of inherited mutations in live or dead offspring; probable human germ cell mutagen (group II), for which data from epidemiological studies to assess germ cell mutagenicity are inadequate: however, there is sufficient evidence of germ cell mutagenicity in animal species (*i.e.*, there is an increased incidence of gene mutations, structural or numerical chromosomal aberrations, or inherited congenital malformations in the live offspring of exposed animals; or an increase in dominant lethal mutations in the potential offspring of exposed animals); possible human germ cell mutagen (group III); unlikely to be a human germ cell mutagen (group IV); probably not a human germ cell mutagen (group V); unclassifiable with respect to germ cell mutagenicity in humans (group VI). Groups III, IV, V and VI have four, two, three and three subgroups, respectively (see Table 5).

The Canadian Environmental Protection Act (13) mentions 'These effects including mutagenic are manifested at the biochemical, cellular, histopathological and morphological levels.', and 'Chemical substances are classified, therefore, with respect to their potential carcinogenicity and mutagenicity to humans; this is accomplished on the basis of rigorous examination of the quantity, quality and nature of the results of available toxicological and epidemiological studies. The criteria by which Priority Substances are classified based on their weight of evidence of carcinogenicity and mutagenicity.'

Health Protection Branch: The Health Protection Branch defined toxicological findings that would be regarded as germ cell genotoxicity (14). The toxicological indication of germ cell genotoxicity are (i) *in vitro* test results and positive evidence for mutagenicity in somatic cells *in vivo*, and (ii) evidence from pharmacokinetic/tissue distribution studies that the test material and/or metabolites reaches the gonads; or (iii) evidence from subchronic or chronic treatment studies that gonadal pathology indicates germ cell damage; or (iv) evidence for reproductive/developmental effects showing reduced numbers of pregnancies, reduced litter sizes or increased time to mating following treatment in some cases. A significant proportion of agents that cause *in vivo* somatic cell mutation might also possess the ability to lead to mutation in germ cells that may be transmitted to offspring. When evidence for *in vivo* somatic genotoxicity is demonstrated, along with tissue distribution, metabolic and/or pathologic evidence that the genotoxic chemical (or metabolites) reaches the germ lines (whether or not overt effects on fertility are found), the possibility of induced genetic damage to germ cells leading to heritable effects should be investigated (14).

Canadian Centre for Occupational Health and Safety: Controlled Products Regulations (CPR) has two mutagenicity criteria (11,15). According to CPR paragraph 57 in the classification of subdivision A (very toxic material), a chemical or chemical mixture is considered to present a hazard to man if (a) there is epidemiological evidence that shows a causal connection between exposure of persons to the substance or mixture and heritable genetic effects; or (b) there is evidence of mutagenicity in mammalian germ cells *in vivo* as shown by (i) positive results in a study that measures mutations transmitted to offspring, or (ii) positive results in an *in vivo* study showing chemical interaction with the genetic material of mammalian germ cells and positive results in an *in vivo* study assessing either gene mutation or chromosomal aberration in somatic cells. CPR paragraph 62 in the classification of subdivision B (toxic material) explains that a pure chemical or a chemical mixture is considered to present a hazard if evidence of mutagenicity in mammalian somatic cells is obtained in a test to assess either gene mutations or chromosomal aberrations.

Japan: The Japanese Industrial Safety and Health Law addresses the mutagenicity classification of substances in the workplace (11). Under this legislation, Japan does not classify mutagenic substances according to a weight of evidence approach or whether a compound may be a somatic or germ cell mutagen but rather recognizes one category: "mutagenic". A substance is classified as mutagenic when the results of a "bacterial reverse mutation assay" are positive. Workers' health impairment by the exposure to the 'strong' mutagenic chemicals or chemical mixtures (excluding those containing 1% or less by weight of mutagenic chemicals), which induce more than 1000 revertants/mg/plate in a bacterial reverse mutation assay, should be prevented in the work for manufacture or handling of these chemicals (16).

Future of Classification of Germ Cell Mutagens

Chemical evaluation of mutagenicity/genotoxicity has three major directions that are (i) screening of carcinogens, (ii) mechanistic investigation of carcinogenesis, and (iii) investigation of heritable adverse effects in germ cells including those in humans. Mutagenicity in GHS is focused on the last topic in terms of classification of germ cell mutagens. GHS and the other existing classification systems have different

Table 5. Criteria for classification of mutagenicity in germ cells in CEPA by Health Canada (13)

Category	Classification	Criteria
Group I	Human Germ Cell Mutagen	Data from adequate epidemiological studies indicate that there is a causal relationship between exposure of humans to a substance and an increased incidence of inherited mutations in live or dead offspring.
Group II	Probable Human Germ Cell Mutagen	Data from epidemiological studies to assess germ cell mutagenicity are inadequate: however, there is sufficient evidence of germ cell mutagenicity in animal species (i.e., there is an increased incidence of gene mutations, structural or numerical chromosomal aberrations, or inherited congenital malformations in the live offspring of exposed animals; or an increase in dominant lethal mutations in the potential offspring of exposed animals).
Group III. A	Possible Human Germ Cell Mutagen	Data from epidemiological studies indicate an association between exposure and human germ cell mutagenicity, but alternative explanations such as chance, bias, or confounding cannot be excluded.
Group III. B	ditto	Data from epidemiological studies to assess germ cell mutagenicity are inadequate: however, there is sufficient evidence of somatic cell mutagenicity (*in vivo* gene mutations or chromosomal aberrations) in humans or animal species, and sufficient evidence of exposure to germ cells in humans or animal species.
Group III. C	ditto	Data from epidemiological studies to assess germ cell mutagenicity in humans are inadequate or lacking. There is sufficient data in animals to indicate that the chemical is a germ cell mutagen, but available data indicate that the induction of mutations occurs through an epigenetic threshold-based mechanism.
Group III. D	ditto	Data from epidemiological studies to assess germ cell mutagenicity in humans are inadequate. There is sufficient evidence of mutagenicity of somatic cells in humans or animal species (*in vivo* gene mutations or chromosomal aberrations), but evidence of exposure to germ cells is inadequate or lacking.
Group IV. A	Unlikely to Be a Human Germ Cell Mutagen	There is no evidence of human germ cell mutagenicity in sufficiently powerful and well-designed epidemiological studies. There is evidence of mutagenicity of somatic cells in well-designed and well-conducted studies in humans or animals, but there is no evidence of exposure of human or animal germ cells in well-designed studies.
Group IV. B	ditto	Data on germ cell mutagenicity in epidemiological studies in humans are inadequate; there is no evidence of mutagenicity *in vivo* in germ or somatic cells in well-designed and properly conducted studies in animals.
Group V. A	Probably Not a Human Germ Cell Mutagen	There is no evidence of germ cell mutagenicity in sufficiently powerful and well-designed epidemiological studies; there is no evidence of germ cell mutagenicity in animal species.
Group V. B	ditto	There is no evidence of germ cell mutagenicity in sufficiently powerful and well-designed epidemiological studies; data in animal species are inadequate.
Group V. C	ditto	Data from epidemiological studies to assess germ cell mutagenicity in humans are inadequate, but evidence of the lack of germ cell mutagenicity in animal species is strongly supported by other data on mutagenicity *in vivo*.
Group VI. A	Unclassifiable with Respect to Germ Cell Mutagenicity in Humans	Data from epidemiological and/or animal studies are inadequate (i.e., because of major qualitative limitations, the studies cannot be interpreted as showing either the presence or absence of germ cell mutagenicity).
Group VI. B	ditto	There are no *in vivo* mutagenicity data available for evaluation.
Group VI. C	ditto	Results of epidemiological studies in human populations and experimental studies in animal species are conflicting, without an identifiable mechanistic basis.

objectives, target audiences and criteria. For example, the primary objective of the systems in GHS, EU and Germany MAK is for hazard classification, on the other hand, the systems of US EPA and Health Canada are for risk assessment. Target audiences are workers in the systems of Germany MAK, Canadian CPR and Japan; consumers and workers in EU system; consumers, workers, transport workers, and emergency responders in GHS. As for criteria, the report from EPA researchers includes adverse effects on reproductive

parameters.

The fundamental purposes are different in these regulations; therefore, it is difficult to discuss the significance of the different approaches. Simple classification category and criteria will be useful for hazard classification. The GHS and EU systems on (germ cell) mutagenicity meet to this point. Application of the criteria needs expert judgment in a weight of evidence approach in GHS. However, the expert judgment leads sometimes different conclusion from expert to expert. This will be a critical issue in the classification of germ cell mutagens by GHS. Furthermore, hazard classification/evaluation is often confused as risk evaluation, especially in Japan. Further efforts including risk evaluation and communication on germ cell mutagenicity will be needed to make understanding of regulations global.

Recently, the European Commission proposed a new regulatory framework for chemicals called REACH for the Registration, Evaluation and Authorisation of Chemicals in October 2003 (17). The authorisation process pays particular attention to the risks that the substance poses due to any carcinogenic, mutagenic, and/or reproductive toxicity (CMR) properties. CMR's category 1 and 2 in the EU criteria, which correspond to the category 1A and 1B, respectively in GHS criteria, are subject to authorization (18,19). All substances imported in quantities over 1 tonne that contain more than 0.1 per cent CMR material must be authorised before gaining access to the EU market. Authorisation provides a permit for specific uses and can be requested by 'producer' or 'user'. The GHS itself is not legally binding, however, some national or regional laws including REACH may be legally binding. Now, classification of germ cell mutagens becomes an important issue.

As GHS criteria have been adopted in worldwide, it will become standard for hazard classification. After implementation of GHS in each country, the classification of chemicals on germ cell mutagenicity will be performed by chemical suppliers (manufacturers or importers). Understanding classification systems for germ cell mutagens will be helpful for scientifically sound classification of chemicals in the GHS.

Acknowledgement: We are grateful to Dr. D.J. Tweats for his review of this manuscript. This work was supported in part by the Health and Labor Sciences Research Grants (H18-Chemistry-General-009).

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Mutagenesis vol. 20 no. 5 pp. 375–379, 2005
Advance Access publication 4 August 2005

doi:10.1093/mutage/gei050

3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone [MX] shows initiating and promoting activities in a two-stage BALB/c 3T3 cell transformation assay

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A transformation assay using BALB/c 3T3 cells was conducted on 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) to assess initiation and promotion activities of MX carcinogenesis. Statistically significant positive responses were obtained compared with the corresponding solvent controls in both the initiation assay post-treated with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and the promotion assay pretreated with 3-methylcholanthrene (MCA). Both TPA and MX inhibited metabolic cooperation in an assay using co-culture of V79 6-thioguanine (6-TG) sensitive and insensitive cells. However, cells isolated from transformed foci in the initiation assay did not induce any nodules after inoculation to BALB/c mice, the strain of mouse from which the transformation assay cells were derived. Although the study was carried out for 2–3 weeks, this might have been too short to develop nodules under the conditions of this experiment. This *in vitro* cell transformation study with MX adds supportive information to studies showing MX carcinogenicity and tumour promoter activity, and adds mechanistic understanding of the action of MX.

Introduction

3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone(MX), an organic chlorinated contaminant commonly found in tap water as a disinfection by-product (1), induces malignant tumours in the rat thyroid and mammary glands (2) and shows promoting activity in the rat stomach (3). Concerning mutagenicity, MX shows positive results in several *in vitro* assays, including the bacterial reverse mutation assay (Ames test) and the cultured cell mutation assay regardless of the presence of an exogenous metabolic activation system. Therefore, MX is classified as a direct acting mutagen (4–9). However, conflicting results have been reported for MX activity in *in vivo* genotoxicity assays (4,5,10–12). We have also performed several *in vivo* assays, namely, detection of 8-hydroxy-deoxyguanosine formation, gene mutation assay using transgenic mice (Muta™Mouse), and the comet assay, and almost all of the assays gave negative responses (M. Nakajima, S. Masumori, M. Kikuchi, S. Inagaki, J. Tanaka, Y. Furuya, M. Hayashi and N. Kinae, manuscript in preparation). Thus, the genotoxicity findings have not provided a conclusive mechanism for MX carcinogenicity.

Among the several types of cell transformation assays, we selected the focus assay using the BALB/c 3T3 established cell line for the present study because of its good reproducibility (13) and because the assay has been widely used in the elucidation of carcinogenicity mechanisms. We used A31-1-1 clone, isolated by Kakunaga and Crow (14) and thought to be sensitive to many chemicals, and employed a protocol with modified culture medium and shortened exposure period (15). The modified method can assess the potential initiation and promotion activities of test chemicals.

Many tumour promoters inhibit gap junctional intercellular communication (GJIC). For example, phorbol esters such as the strong promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA) inhibit GJIC in metabolic cooperation assays (16–18). As such, the metabolic cooperation assay has been widely used as a tool for detection of promoters and for providing information about the mechanism of carcinogenicity. Therefore, we applied the assay using V79 cells to assess the promoting potential of MX.

Materials and methods

Test compounds and positive control substance

MX (CAS No. 77439-76-0, purity: 99.2%) was synthesized at the Laboratory of Food Hygiene, University of Shizuoka according to a modified method (19). It was dissolved in physiological saline, Japanese Pharmacopoeia (Otsuka Pharmaceutical Factory, Inc.). 3-Methylcholanthrene (CAS No. 56-49-5; MCA; Wako Pure Chemical Industries Ltd, Osaka, Japan), the initiator, was dissolved in dimethylsulfoxide (DMSO; purity: 99.7% or higher: Merck KGaA, Darmstadt, Germany) and used at a concentration of 0.2 μg/ml. TPA (CAS No. 16561-29-8; Wako Pure Chemical Industries Ltd), the promoter, was dissolved in DMSO and used at 0.1 μg/ml.

Preparation of test cells and culture medium

BALB/c 3T3 A31-1-1 cells were supplied by Showa Denko K.K. Cells were cultured in a CO_2 incubator (FORMA and SANYO Electric Medica Systems Co. Ltd) under 5% CO_2 atmosphere at 37°C. Eagles-MEM liquid medium (Asahi Techno Glass Corporation, Funabashi, Chiba) supplemented with 10% fetal bovine serum (FBS; Moregate BioTech, Bulimba, Australia) and 60 mg/ml Kanamycin sulfate (Invitrogen Corp., NY) (MEM) was used throughout the experiment unless otherwise indicated. Low serum concentration medium (DMEM:F12) used was DMEM:HAM's F-12 liquid medium (Asahi Techno Glass Corporation) supplemented with 5.2 ml Daigo's ITES (insulin, transferrin, ethanolamine, sodium selenite; Wako Pure Chemical Industries Ltd) per 500 ml and 2% FBS. The 500 ml extracting medium for absorption measurement was composed of 4.48 g sodium citrate dihydrate, 97.5 ml of 0.1 mol/l HCl, and 250 ml ethanol made to 500 ml with distilled water. For the metabolic cooperation assay using V79 cells, the medium used was Eagles-MEM liquid medium supplemented with 3% fetal bovine serum, 0.1% Eagle's non-essential amino acids (Invitrogen Corp.), 0.1% pyruvic acid and 0.1% glutamic acid.

Dose range-finding cytotoxicity test

For cytotoxicity testing with the initiation assay protocol, 1×10^3 cells were seeded into 24-well plates and treated 24 h later with MX at 1.42, 1.90, 2.53,

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3.38, 4.50, 6.00 and 8.00 μg/ml. The culture medium was removed 72 h later, and the cells were fixed with 10% neutral buffered formalin and stained with 0.1% crystal violet for 30 min. For cytotoxicity testing with the promotion assay protocol, cells were seeded as above and MEM was replaced with DME/F12 medium 48 h after seeding. The cells were then treated with 1.68, 2.10, 2.62, 3.28, 4.10, 5.12 and 6.40 μg/ml of MX, and fixed and stained 96 h after cell seeding in the same manner as for the initiation assay cytotoxicity test. Extractant (1.5 ml) was placed in each well for 10 min and then absorption was measured with a spectrophotometer set at 580 nm. Cell survival at each dose was calculated relative to the negative vehicle control group. The MX concentration that inhibited cell growth by 50% (IC_{50} value) was calculated using the Probit method and approximately twice the IC_{50} was selected as the highest concentration for the cell transformation assays.

Cell transformation assays

In the initiation assay, 1.2×10^4 cells were seeded into 60 mm diameter culture dishes, 12 dishes per concentration, and control groups. After 24 h incubation, DMSO as negative control, MX as the initiator (1.64, 2.05, 2.56, 3.20 and 4.00 μg/ml) or MCA as a positive control (0.2 μg/ml) was added. Seventy-two hours after treatment, MEM was replaced with fresh DME/F12. On the 7th day after the beginning of treatment, TPA (0.1 μg/ml) or DMSO was added to cultures as the first promoter treatment. For the second and third promoter treatments, TPA or DMSO was added on the 11th and 14th day, respectively.

In the promotion assay, MCA (0.2 μg/ml, as the initiator) or DMSO was added 24 h after seeding 1.2×10^4 cells per dish. On the 4th day, MEM was replaced with fresh DME/F12. On the 7th day, saline, MX (0.156, 0.313, 0.625, 1.25 and 2.50 μg/ml) or TPA (positive control; 0.1 μg/ml) was added to the culture. MX and TPA were also added on the 11th and 14th day.

The cells were fixed with methanol and stained with 2.5% Giemsa solution on the 25th day for both assays. The foci that met the following criteria were counted as transformant: (i) 2 mm or more in diameter, (ii) criss-cross growth pattern, (iii) layering of cells and (iv) deep basophilic staining.

Tumourigenicity of transformed cells

Six-week-old male BALB/c CR mice were purchased from Japan Slc, Inc. (Shizuoka, Japan), and were quarantined and acclimated to the testing facility for 1 week. They were given pelleted diet (MF: Oriental Yeast Co., Ltd) and tap water *ad libitum* through the acclimation and assay periods.

At the end of the transformation assay with MX (4.0 μg/ml as initiator and 2.5 μg/ml as promoter; experimental data not shown), cultures were washed once with Dulbecco's PBS. Cells were isolated from transformed foci by trypsinization and mass cultured. An aliquot of 0.2 ml of cell suspension (1×10^6 cells for Experiment 1 and 1.5×10^6 cells for Experiment 2) was injected subcutaneously into the cervical region of the BALB/c CR mice. In both experiments, the cells isolated from the transformed foci in the negative control groups were inoculated into three animals and cells isolated from MX-induced transformed foci were inoculated into four animals. All animals were examined 2 weeks (Experiment 1) or 3 weeks (Experiment 2) after inoculation.

Metabolic cooperation assay

6-Thioguanine (6-TG) sensitive V79 cells (6-TG^s; 4×10^5 cells) and 6-TG resistant cells (6-TG^r; 200 cells) were co-cultured to evaluate the inhibition of metabolic cooperation (5 dishes per concentration). For calculating cytotoxicity 200 V79 [6-TG^s or 6-TG^r] cells alone were plated (3 dishes per concentration). Cells were treated with either MX or TPA 4 h after seeding. The 6-TG (10 μg/ml) was added 15 min after MX or TPA treatment and cells cultured for an additional 3 days before the medium was replaced with fresh medium containing only 6-TG; the cells were cultured another 4 days. The cells were fixed in ethanol and stained with 0.1% crystal violet for 10 min. Colonies with 50 or more cells were counted. These colonies developed from cells that were either not in GJIC contact with 6-TG^s cells or were in contact but then 'rescued' by GJIC inhibition from test chemical action. The assay is based on toxicity of 6-TG to 6-TG^{s}V79 cells (HGPRT$^+$), non-toxicity of 6-TG to 6-TG^r mutant V79 cells (HGPRT$^-$), with toxicity to these latter cells if in GJIC contact with HGPRT$^+$ cells, which transfer the HGPRT-catalysed toxic 6-TG metabolite via gap junctions to the HGPRT$^-$ cells. Inhibition of GJIC rescues the contacting mutant cells to allow their clonal expansion (20).

Statistical analysis

The percentage of dishes with foci and the mean number of foci per dish were analysed using Fisher's exact test and the Wilcoxon's rank sum test, respectively.

In the metabolic cooperation assay, the number of 6-TG^r colonies was analysed for difference from the negative control group using Dunnett's test.

Results

Initiation assay

In the cytotoxicity assay for dose-range finding, a concentration-dependent decrease in cell survival was observed with MX treatment (Figure 1). An IC_{50} value using Probit's method was calculated to be 1.92 μg/ml. In the negative control group (saline initiation–TPA promotion) the mean number of transformed foci per dish was 0.50 and the percentage of dishes with foci was 41.7% (5 of 12 dishes, Table I). When MX was used as an initiator at 1.64, 2.05, 2.56, 3.20 and 4.00 μg/ml, and with DMSO post-treatment, no significant increase in transformation was observed. In the five groups treated with 1.64–4.00 μg/ml MX (as initiator) and TPA (0.1 μg/ml, as promoter) the numbers of foci per dish were

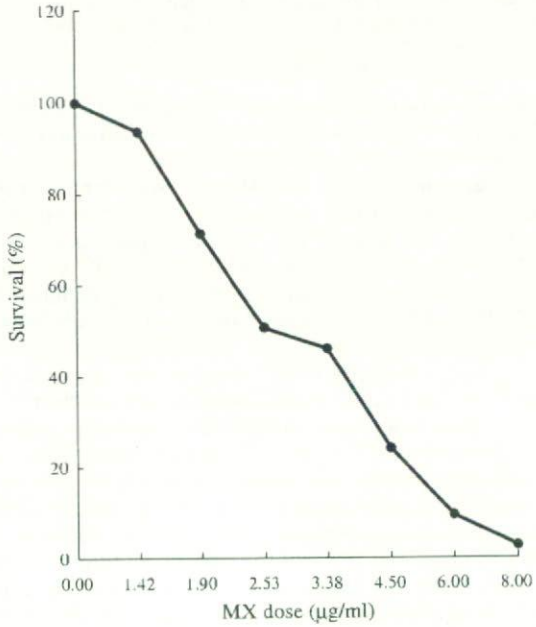

Fig. 1. MX dose–response for cell survival (Initiation protocol).

Table I. Initiating activity of MX in the two-stage transformation assay

Initiator	Conc. (μg/ml)	Survival (%)	Promoter	Conc. (μg/ml)	Mean foci/dish	Number of dishes with foci
Saline		100.0	DMSO		0.09	1/11 (9.1%)
MX	1.64	73.8	DMSO		0.08	1/12 (8.3%)
	2.05	59.2			0.25	2/12 (16.7%)
	2.56	33.7			0.00	0/12 (0.0%)
	3.20	20.5			0.33	3/12 (25.0%)
	4.00	18.8			0.25	3/12 (25.0%)
MCA	0.2	57.6	DMSO		0.58*	7/12* (58.3%)
Saline		–	TPA	0.1	0.50	5/12 (41.7%)
MX	1.64	–	TPA	0.1	0.58	6/12 (50.0%)
	2.05	–			1.25	8/12 (66.7%)
	2.56	–			1.92*	10/12 (83.3%)
	3.20	–			1.82*	9/11 (81.8%)
	4.00	–			3.64**	11/11** (100%)
MCA	0.2	–	TPA	0.1	7.58**	12/12** (100%)

*$P \leq 0.05$, **$P \leq 0.01$, significant difference from control (Wilcoxon's rank sum test for mean number of foci and Fisher's exact test for the percentage of dishes with foci).
Conc., concentration.

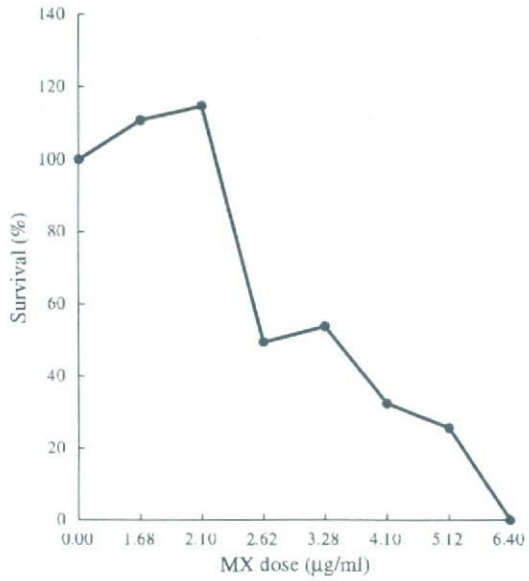

Fig. 2. MX dose–response for cell survival (Promotion protocol).

0.58, 1.25, 1.92, 1.82 and 3.64, respectively. A statistically significant increase ($P < 0.05$) was observed at MX ≥2.56 μg/ml compared with the negative control group. The number of dishes with foci also increased in a concentration-dependent manner and foci were observed in all dishes at the highest concentration (4.00 μg/ml) group. A large number of foci were induced in the positive control and MCA initiation-TPA promotion group; the mean number of foci per dish was 7.58, and all dishes contained foci.

Promotion assay

In the cytotoxicity assay, a concentration-dependent decrease in cell survival with MX treatment was observed (Figure 2). The IC_{50} value was 3.37 μg/ml. The experimental doses for promotion assay included at least 2 doses expecting to have ≥90% cell survival rate. In the negative control group (MCA initiation–saline promotion), the mean number of foci per dish was 0.25 and the percentage of dishes with foci was 25% (3 out of 12 dishes, Table II). After initiation treatment with DMSO, cells were treated with MX at 0.156, 0.313, 0.625, 1.25 and 2.50 μg/ml as the promoter. The numbers of foci were 0.00, 0.08, 0.00, 1.10 and 0.90 per dish, respectively, with only the highest dose eliciting a significant increase in dishes with foci (Table II). In the groups treated with MCA (0.2 μg/ml) and MX at the above five concentrations, the numbers of foci were 0.25, 0.92, 1.33, 3.40 and 6.18 per dish, respectively. A statistically significant increase ($P < 0.05$) was observed at concentrations ≥0.625 μg/ml compared with the negative control group. The number of dishes with foci also increased concentration-dependently, and foci were observed in all dishes of the 1.25 and 2.50 μg/ml groups. The positive control group (MCA initiation–TPA promotion) confirmed the effectiveness of MCA/TPA in this cell transformation assay.

Tumourigenicity assay

Gross examination of the mice necropsied in tumourigenicity experiments 1 and 2 did not reveal any visible nodules or tissue masses in any organs of any animals.

Table II. Promoting activity of MX in the two-stage transformation assay

Initiator	Conc. (μg/ml)	Promoter	Conc. (μg/ml)	Survival (%)	Mean foci/dish	Number of dishes with foci
DMSO		Saline		100.0	0.08	1/12 (8.3%)
DMSO		MX	0.156	98.5	0.00	0/12 (0.0%)
			0.313	103.2	0.08	1/12 (8.3%)
			0.625	93.3	0.00	0/12 (0.0%)
			1.25	90.6	1.10	4/10 (40.0%)
			2.50	90.2	0.90**	7/10** (70.0%)
MCA	0.2	Saline		–	0.25	3/12 (25.0%)
DMSO		TPA	0.1	160.8	0.25	3/12 (25.0%)
MCA	0.2	MX	0.156	–	0.25	3/12 (25.0%)
			0.313	–	0.92	5/12 (41.7%)
			0.625	–	1.33*	10/12** (83.3%)
			1.25	–	3.40**	10/10** (100%)
			2.50	–	6.18**	11/11** (100%)
MCA	0.2	TPA	0.1	–	2.73**	10/11** (90.9%)

*$P \leq 0.05$, **$P \leq 0.01$, significant difference from control (Wilcoxon rank sum test for mean number of foci and Fisher's exact test for the percentage of dishes with foci).
Conc., concentration.

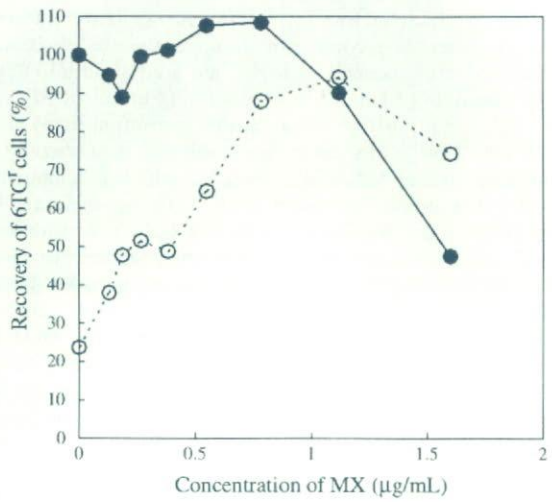

Fig. 3. Inhibition of intercellular metabolic cooperation in V79 cells by MX. For calculation of cell survival, 200 6-TG^r cells were cultured (filled circle). Recovery of 6-TG^r cells (open circle) under conditions of metabolic cooperation (4×10^5 6-TG^s cells and 200 6-TG^r cells).

Inhibition of metabolic cooperation assay

The mean number of 6-TG^r colonies increased dose-dependently in the MX-treated groups (Figure 3), indicating an inhibition of GJIC by MX. This occurred at non-cytotoxic concentrations. The mean number of 6-TG^r colonies at 1.12 μg/ml of MX was 188.4 (=94.2%) compared with 47.6 (=23.8%) in the negative control. In the positive control (TPA-treated) group the mean number of 6-TG^r colonies was 159.4 (=79.7%).

Discussion

From a public health viewpoint it is important to understand the toxicology of MX. This is particularly evident since long-term animal studies have shown carcinogenic and tumour promoting activity of MX (2,3). In the present study, we conducted transformation assays on MX using BALB/c 3T3 cells to give