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

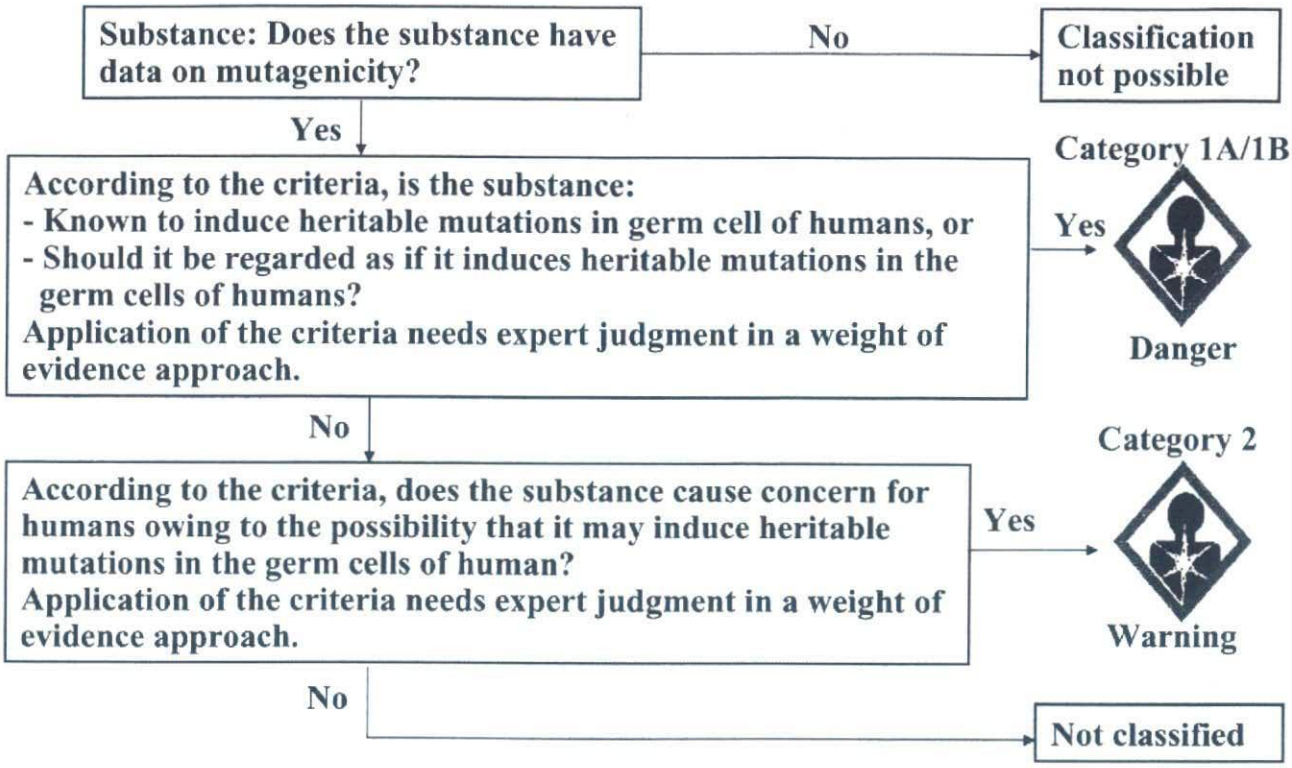

Fig. 1. Decision logic for the classification of germ cell mutagens in GHS (2)

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.

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References

1 United Nations (UN). Globally Harmonized System of Classification and Labelling of Chemicals (GHS), New York and Geneva, 2003. (http://www.unece.org/trans/danger/publi/ghs/ghs_rev00/00files_e.html)

2 United Nations (UN). Globally Harmonized System of Classification and Labelling of Chemicals (GHS), First revised edition, New York and Geneva, 2005. (http://www.unece.org/trans/danger/publi/ghs/ghs_rev01/01files_e.html)

3 Japanese inter-ministerial committee for GHS. Japanese translation version of the first revised edition of GHS documents, 2005. (in Japanese). (http://www.meti.go.jp/policy/chemical_management/kokusai/GHS/GHStexts/kariyaku.htm)

4 Japanese inter-ministerial committee for GHS. Publication of classification of chemicals under each relevant domestic law (in progress), 2006. (in Japanese). (http://www.safe.nite.go.jp/ghs/index.html)

5 European Communities (EC). Commission directive 2001/59/EC of 6 August 2001, Official J. of Europe Commun. L225, 21.8.2001.

6 Pratt IS, Barron T. Regulatory recognition of indirect genotoxicity mechanisms in the European Union. Toxicol Lett. 2003; 140–141: 53–62.

7 European Communities (EC). Classification, packaging, and labeling of dangerous substances in the EU, Part I, Office for Official Publications of the European Communities, Luxembourg, 1997.

8 Adler ID, Anrae U, Kreis P, Neumann HG, Their R, Wild D. Recommendation for the categorization of germ cell mutagens. Int Arch Occup Environ Health. 2000; 73: 428–32.

9 Deutsche Forschungsgemeinschaft (DFG). List of MAK and BAT values, Commission for the investigation of health hazards of chemical compounds in the work area, Report No. 39, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany; 2003.

10 US Environmental Protection Agency (USEPA). Guidelines for Mutagenicity Risk Assessment, Federal Register 51(185):34006-34012, EPA/630/R-98/003; September 1986.

11 Organisation for Economic Co-operation and Development (OECD). OECD Series on testing and assessment, Number 12, Detailed Review Document on Classification Systems for Germ Cell Mutagenicity in OECD Member Countries, ENV/JM/MONO(99)2, Environment Directorate, OECD, Paris, 1999.

12 Dearfield KL, Cimino MC, McCarroll NE, Mauer I, Valcovic LR. Genotoxicity risk assessment: a proposed classification strategy. Mutat. Res. 2002; 26: 121–35.

13 Health Canada. Canadian Environmental Protection Act (CEPA). Human Health Risk Assessment for Priority Substances, Minister of Supply and Services Canada, Ottawa, Canada, Cat. No. En40-215/41E; 1994.

14 Health Protection Branch. The assessment of mutagenicity, Mutagenicity Guidelines, Ministry of Supply and Services Canada, Cat. H49-85/1993E; 1993.

15 Canadian Centre for Occupational Health and Safety (CCOHS). Controlled Products Regulations. SOR/88-66; 1988.

16 Guidelines for Preventing Health Impairment by Chemical Substances with Mutagenicity Recognizes, LSB Notification No. 312, Industrial Safety and Health Law, Japan, dated May 17, 1993.

17 European Commission. Proposal for a Regulation of the European parliament and of the council concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency and amending Directive 1999/45/EC and Regulation (EC), COM(2003) 644 final, Brussels; 2003.

18 European Commission Services. Comparison between EU and GHS criteria, Human health and environment (Draft), Version June 08, 2005.

19 Morita T, Ishimitsu S, Morikawa K. Genotoxicity in risk assessment, Global perspectives. Environ. Mutagen Res. 2005; 27, 47-56. (in Japanese)

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SFTG international collaborative study on in vitro micronucleus test III. Using CHO cells

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Abstract

In this report, results are presented from an international study of the in vitro micronucleus assay using Chinese hamster ovary cells. This study was coordinated by an organizing committee supported by the SFTG (the French branch of the European Environmental Mutagen Society). Test chemicals included mannitol, bleomycin, cytosine arabinoside, urethane and diethylstilboestrol. Mitomycin C was used as a positive control. Each chemical was evaluated in at least two laboratories following a variety of different protocols (short and long exposures, varying recovery times, with and without cytochalasin B) in order to help determine a standard protocol for routine testing in Chinese hamster ovary cells. Mannitol and urethane were negative, while bleomycin, cytosine arabinoside and diethylstilboestrol induced a dose dependent increase in micronucleated cells. In the presence of cytochalasin B, increases in micronuclei were observed in binucleated as well as mononucleated cells in cultures treated with bleomycin, cytosine arabinoside or diethylstilboestrol. Importantly, all three of these chemicals were detected in each of the different treatment/recovery regimens. No differences were seen in the sensitivity or accuracy of the responses in the presence of absence of cytochalasin B. Overall, these results demonstrate the suitability of Chinese hamster ovary cells for the in vitro micronucleus assay.

Keywords: In vitro micronucleus assay; CHO cells; Cytochalasin B; Clastogen; Aneugen

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1. Introduction

Established cell lines, especially those developed from Chinese hamsters (CHO-WBL, CHO-K1, V79, CHL) are commonly used in the standard metaphase chromosome aberration assay (e.g. Ishidate et al. [1]). Chinese hamster cell lines have the advantage of having a relatively small number of chromosomes (20–22 chromosomes), they are readily available, they do not have donor variability like human lymphocytes, and they do not require mitogenic stimulation for growth since most of the cells are dividing. Based on their extensive use in chromosome aberration assays, Chinese hamster cell lines have increasingly been used for the assessment of micronuclei in vitro [2–15]. Not unexpectedly, various protocols have emerged for the in vitro micronucleus assay in Chinese hamster cells and there are few studies that have carefully examined the critical parameters of the protocol. Before an internationally harmonized protocol can be developed for the in vitro micronucleus assay using Chinese hamster cell lines, agreement on the key aspects of the protocol is needed [16,17].

To address this, an international study starting in 1997 was coordinated by an organizing committee supported by the SFTG (the French branch of the European Environmental Mutagen Society) and included laboratories from Europe, America and Japan. As part of this, studies in Chinese hamster ovary cells were conducted with a common protocol. Since the initiation of these studies, Garriott et al. [2] and Phelps et al. [18] reported the results of studies in Chinese hamster ovary cells with a total of 26 chemicals for both, complementary to this present study and that have also a variety of mechanisms of genotoxicity including aneugens, crosslinking agents, and strand breaking agents. They investigated important parameters such as the use of cytochalasin B, length of exposure, number of cells to analyse. The outcome of the present international study, together with these previous papers will contribute to a defined standard protocol for the use of in vitro micronucleus test for routine genotoxicity testing in Chinese hamster ovary cells.

2. Materials and methods

2.1. Cells

CHO-WBL cells were originally obtained from Covance Laboratories (Vienna, VA, USA), Abbott Laboratories (Abbott Park, IL, USA) or BioReliance (Rockville, MD, USA).

2.2. Culture media

Mc Coy's 5A (with bicarbonate buffer and Hepes) supplemented with penicillin–streptomycin (50 UI/ml–50 μg/ml), glutamine (2 mM) and 10% heat-inactivated fetal calf serum.

2.3. Chemicals

The test chemicals were purchased from Sigma Chemicals, coded and dispatched to the participants of the study by the organizing committee (see the general publication in this issue for details [19]).

They were: mannitol (CAS No. 69-65-8), bleomycin (CAS No. 9041-93-4), cytosine arabinoside (CAS No. 147-94-4), urethane (CAS No. 51-79-6) and diethylstilboestrol (CAS No. 56-53-1). Mitomycin C (CAS No. 50-07-7) was used as the positive control.

2.4. Culture conditions

A survey was performed before the study to define the procedures in use in the different participating laboratories and to elaborate a common protocol based on these current practices. Cultures were performed in duplicate. In some studies with cytochalasin B, a separate third culture flask was used for cell counts to provide a complementary evaluation of cytotoxicity to compare to the ratio of binucleated cells. Following the current use validated in each laboratory, different conditions were applied for seeding cells. The day before treatment, the cells were seeded in either 4-well chambers, 8-well chambers, Petriperm chambers, 6-well plates or 35-mm dishes. Cells were cultured at densities of 10,000–14,000 cells/cm^2 in the '3 + 20 h' schedule with cytochalasin B; 2400–5000 cells/cm^2 in the '3 + 45 h' or '24 + 24 h' schedules without cytochalasin B; 2400–7000 cells/cm^2 in the '24 + 20 h' schedule with cytochalasin B or '24 + 0 h' and '3 + 21 h' schedules without cytochalasin B. In these conditions, cells were actively growing upon treatment.

All cultures were incubated at 37 °C in a humidified atmosphere of approximately 5% CO_2 and filtered air.

2.5. Treatment and recovery times

The different treatment and recovery schedules are summarised in Fig. 1.

Cells were treated with the test compound by replacing the culture medium with fresh treatment medium containing various concentrations of the test compound. The cultures were incubated for the required treatment period (3 or 24 h) after which the treatment medium was removed and the cells were rinsed with buffer or culture medium. One set of cultures was harvested immediately after the 24 h treatment with no recovery period ('24 + 0 h'). The cultures that were not harvested immediately after rinsing were then re-fed with either fresh culture medium without cytochalasin B or with culture

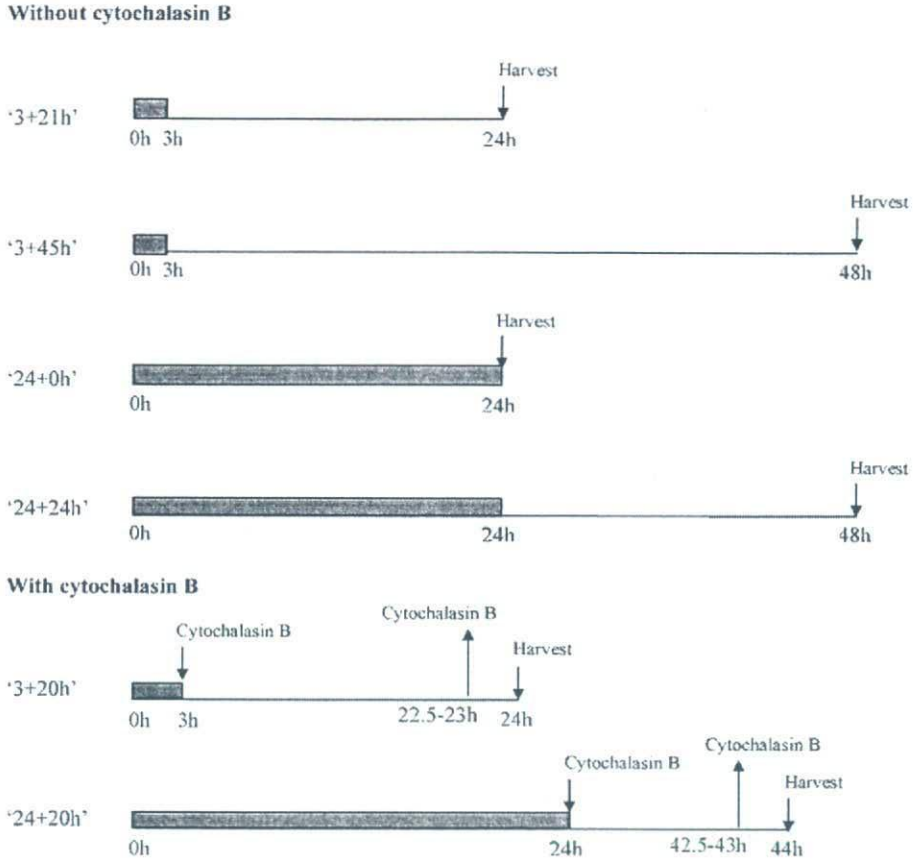

Fig. 1. Treatment and recovery schedules: (■) treatment with a chemical; (—) recovery in the fresh medium.

medium containing cytochalasin B. For recovery in the absence of cytochalasin B, cells were incubated either 21 or 45 h following the 3 h treatment period ('3 + 21' or '3 + 45') or for 24 h following the 24 h treatment ('24 + 24 h') and harvested. For recovery in the presence of cytochalasin B, cells were incubated either 20 h or 45 h following the 3-h treatment period ('3 + 20 h or '3 + 45 h') or incubated for 20 h following the 24 h treatment ('24 + 20 h').

Cytochalasin B, when used, was added to the cultures at a final concentration of 3 μg/ml in the medium, after the test compound had been removed. Cells were allowed to recover from cytochalasin B for an additional 1–1.5 h prior to harvest to improve cellular morphology and micronucleus scoring. For instance, cells treated with the test chemical for 3 h followed by a 20-h recovery in the presence of cytochalasin B were harvested around 21 h after completion of treatment (24 h from the start of chemical exposure). From the experience of the participants collected during the survey, this step was known to be necessary to obtain nice preparations for these cells.

Mitomycin C was used as the positive control at concentrations of 0.1 μg/ml for all of the 24-h treatments and at 0.2 or 0.5 μg/ml for the 3-h treatments. The concentrations were determined following a preliminary positive control dose range-finding study in all the participating laboratories (see the main publication in this issue [19]).

2.6. Cell sampling and staining

At the appropriate harvest time (see Fig. 1), the cells were rinsed and exposed to a hypotonic shock in situ using either 75 mM KCl or 1% (w/v) sodium citrate. The cells were then fixed with methanol:acetic acid (3:1) and/or 100% (v/v) methanol, and stained with either giemsa or acridine orange.

2.7. Report and evaluation of results

In cultures that were exposed to cytochalasin B, the incidence of micronucleus formation was assessed by counting the number of micronucleated binucleated cells in 1000 binucleated cells per culture (2000 cells per test compound concentration). The incidence of micronucleated mononucleated cells in 1000 mononucleated cells per culture was also analysed, when possible, in the cultures exposed to cytochalasin B. In cultures that were not exposed to cytochalasin B the incidence of micronucleated cells in 1000 cells per culture was analysed (2000 cells per test compound concentration).

Criteria for assay acceptability and interpretation of results are described in the general publication in this issue [19]. Table 1 summarises the assays that were used for this evaluation. The range of tested concentrations is reported for each assay with the highest negative concentration or the lowest positive concentration as well as the corresponding survival percentage and maximal fold increase (MFI), where applicable. The survival percentage was calculated relative to the solvent control and is based on cell counts after treatment when cytochalasin B was not used or on the percentage of binucleated cells when cytochalasin B was used. The fold increase was calculated by dividing the number of micronucleated cells in the treated cultures by the number of micronucleated cells in the solvent control cultures. Data for individual cultures are reported in Appendix A.

The criteria for a positive response were the demonstration of a significant, reproducible, and concentration-dependent increase in the number of micronucleated cells, at one or more test compound concentrations, relative to the number of micronucleated cells in the solvent control. If all of these criteria were not achieved the test compound response was equivocal, and if none of the criteria was achieved the test compound response was negative.

3. Results

3.1. Spontaneous background and mitomycin C-induced responses

The spontaneous background of micronucleated cells among laboratories was around 12 per 1000 cells, whatever the treatment schedule, and ranged from 6 to 27 per 1000 cells. This variability between laboratories was also seen in other cell types in this collaborative study (see the general publication in this issue [19] where the data were detailed). This was also reported for human lymphocytes by the HUMN work [20] where the micronucleus identification was recognised as the main source of variability. However, in most cases, the highest was the negative control, the highest was the induced response. Therefore, this variability was not misleading for the test compounds evaluation but may account for non-reproducible sporadic responses (e.g. for urethane).

The influence of cytochalasin B on spontaneous and mitomycin C-induced numbers of micronucleated cells was also considered. Theoretically, for a given treatment schedule, the incidence of micronucleated cells in binucleates in the presence of cytochalasin B should be divided by two to be compared to the incidence of micronucleated cells in mononucleates in the presence of cytochalasin B. This expected excess of micronucleated cells in the presence of cytochalasin B was actually seen for mitomycin C-induced numbers of micronucleated cells (146 with versus 102 per 1000 without cytochalasin B for the 3 h treatment and 382 with versus 183 per 1000 without cytochalasin B for the 24-h treatment). Nevertheless, the ratio for induced levels was below the theoretical two-fold ratio, and this excess of micronucleated cells in the presence of cytochalasin B was not seen for spontaneous levels. This may be due to a partial inefficiency of cytochalasin B and was probably related to the stage of the cell cycle when the primary lesion leading to a micronucleus occurred. However, this had no consequence on the genotoxic evaluation of the compounds.

3.2. Mannitol

There was no reproducible cytotoxicity, where evaluated, up to 5000 μg/ml. In one laboratory a precipitate was found at 1000 or 5000 μg/ml. In one assay in the presence of cytochalasin B, after the 24 h treatment, the number of micronucleated cells was statistically significantly higher in the treated cultures than in the control. However, this slight increase (1.6 to 2.6-fold the control values), not reproduced in the second assay was considered irrelevant. No statistically significant increases in the number of micronucleated cells were seen in all other assays. Therefore, the compound was concluded negative regardless the treatment schedule used.

3.3. Bleomycin

Bleomycin induced concentration-dependent and reproducible increases in the number of micronucleated cells. These effects were found at concentrations inducing little or no cytotoxicity and were distributed over a wide concentration range (up to a one log difference between lowest and highest positive concentrations).

Surprisingly, in the presence of cytochalasin B, an induction of micronucleated cells was also found in mononucleated cells, in addition to binucleated cells, although to a lesser extent. The incidence of micronucleated cells was generally higher in binucleated cells and was more pronounced after the long treatment. This increase of the number of micronuclei in mononucleated cells, previously reported for aneugens was not expected with bleomycin [21]. Since bleomycin is able to induce DNA damage at every stage of the cell cycle, these micronucleated mononucleated cells may represent the cells that had completed the division before the exposure to cytochalasin B. The difference of micronucleated cell numbers between mononucleated and binucleated cells

Table 1
Summary data with CHO cells

Compound	CAS No.	Schedule	Cyt B[a]	Lab.	Assay	Range of concentrations (μg/ml)[b]	Highest negative concentration (μg/ml)[c]		Lowest positive concentration (μg/ml)[c]		Maximal fold increase of the range[g]	Conclusion for the assay[h]	Judgement for the treatment and recovery schedule[i]
							Result and concentration	Survival (%)[d]	Result and concentration	Survival (%)[f]			
Mannitol	69-65-8	3 + 21	–	1	1	500–5000 p	−5000	nt				–	Negative
				1	2	100–1000 p	−1000	80				–	
				2	1	78–5000	−5000	≥100				–	
				2	2	1250–5000	−5000	≥100				–	
		3 + 45	–	1	1	100–1000 p	−1000	48				–	Negative
				1	2	100–1000 p	−1000	98				–	
				2	1	78–5000	−5000	89				–	
				2	2	1250–5000	−5000	≥100				–	
		24 + 0	–	1	1	100–1000 p	−1000	47				–	Negative
				1	2	100–1000 p	−1000	≥100				–	
				2	1	78–5000	−5000	≥100				–	
				2	2	1250–5000	−5000	87				–	
		24 + 24	–	1	1	500–5000 p	−5000	nt				–	Negative
				1	2	100–1000 p	−1000	≥ 100				–	
				2	1	78–5000	−5000	76				–	
				2	2	1250–5000	−5000	92				–	
		3 + 20	+	1	1	500–5000 p	−5000	83				–	Negative
				1	2	100–1000 p	−1000	≥100				–	
				2	1	78–5000	−5000	≥100				–	
				2	2	1250–5000	−5000	99				–	
		24 + 20	+	1	1	500–5000 p			+500 b	≥100 b	2.6 b	+	Negative
				1	2	100–1000 p	−1000	87				–	
				2	1	78–5000	−5000	≥100				–	
				2	2	1250–5000	−5000	≥100				–	
Bleomycin	9041-93-4	3 + 21	–	1	2	62.5–500			+62.5	69	10.4	+	Positive
				2	1	0.11–500			+0.45	77	15.1	+	
				2	2	15.6–250			+15.6	75	17.7	+	
		3 + 45	–	1	1	0.88–250			+0.88	91	8.0	+	Positive
				1	2	0.88–250			+0.88	93	9.5	+	
				2	1	0.11–500			+0.45	≥100	10.8	+	
		24 + 0	–	1	1	3.5–500			+3.5	77	5.7	+	Positive
				1	2	3.5–500			+3.5	62	6.8	+	
				2	1	0.11–500			+ 0.11	81	26.7	+	
		24 + 24	–	1	2	3.5–125			+3.5	63	11.5	+	Positive
		3 + 20	+	1	1	0.17–14			+0.17 m, b	70 m, b	1.9 m, 8.5 b	+	Positive
				1	2	3.5–500			+3.5 m, b	76 m, b	2.0 m, 6.1 b	+	
				2	1	0.11–62.5			+0.11 b[j]	≥100 b	51.5 b	+	
				2	2	10–100			+10 b[j]	78 b	13.7 b	+	
		24 + 20	+	1	1	0.17–0.875			+0.17 m, b	≥100 m, b	16.3 m, 22.1 b	+	Positive
				1	2	0.29–3.5			+0.29 m, b	93 m, b	11.1 m, 15.5 b	+	

Table 1 (*Continued*)

Compound	CAS No.	Schedule	Cyt B[a]	Lab.	Assay	Range of concentrations (μg/ml)[b]	Highest negative concentration (μg/ml)[c]		Lowest positive concentration (μg/ml)[e]		Maximal fold increase of the range[g]	Conclusion for the assay[h]	Judgement for the treatment and recovery schedule[i]
							Result and concentration	Survival (%)[d]	Result and concentration	Survival (%)[f]			
				2	1	0.0017–7.8			+0.0068 b[j]	90 b	50.4 b	+	
				2	2	0.0025–0.1			+0.025 b[j]	≥100 b	6.1 b	+	
Cytosine arabinoside	147-94-4	3+21	–	1	1	0.03–100			+3	76	7.8	+	Positive
				1	2	1–30			+3	75	5.2	+	
				2	1	0.625–20			+1.25	76	30.7	+	
				2	2	2.5–10			+2.5	≥100	7.3	+	
		3+45	–	1	1	0.03–100			+3	76	2.1	+	Positive
				1	2	1–30			+3	67	2.8	+	
				2	1	0.625–20			+5	64	5.4	+	
				2	2	5–30			+5	77	31.6	+	
		24+0	–	1	1	0.01–10			+0.1	50	5.2	+	Positive
				1	2	0.03–1			+0.1	72	8.5	+	
				2	1	0.0098–0.3125			+0.156	62	11.4	+	
				2	2	0.078–0.625			+0.078	67	15.3	+	
		24+24	–	1	1	0.01–10			+0.1	66	3.2	+	Positive
				1	2	0.03–1			+0.1	69	13.6	+	
				2	1	0.0098–0.3125			+0.0195	≥100	8.5	+	
				2	2	0.0098–0.3125			+0.078	80	15.4	+	
		3+20	+	1	1	0.3–100			+3 b	≥100 b	7.8 b	+	Positive
				1	2	1–30			+10 m, + 3 b	67 m, ≥100 b	9.0 m, 8.2 b	+	
				2	1	0.625–20			+20 m, 1.25 b	44 m, ≥100 b	12.0 m, 7.1 b	+	
				2	2	2.5–10			+2.5 m, b	≥100 m, b	7.3 m, 11.8 b	+	
		24+20	+	1	1	0.003–1			+0.1 m, b	≥100 m, b	12.5 m, 49.1 b	+	Positive
				1	2	0.03–1			+0.1 b	≥100 b	34.8 b	+	
				2	1	0.0098–0.3125			+0.078 m, b	≥100 m, b	58.3 m, 20.7 b	+	
				2	2	0.039–0.3125			+0.039 m, b	≥100 m, b	422.9 m, 42.7 b	+	
Urethane	51-79-6	3+21	–	1	1	1000–5000	−5000	59				–	Negative
				1	2	1000–5000	−5000	71				–	
				2	2	625–5000	−5000	85				–	
				3	1	1250–5000	−5000	83				–	
		3+45	–	1	1	1000–5000	−5000	≥100				–	Negative
				1	2	1000–5000	−5000	60				–	
				3	1	1250–5000	−5000	95				–	
		24+0	–	1	1	1000-5000	−5000	82				–	Negative
				2	2	625–5000	−5000	63				–	
				3	1	1250–5000	−5000	71				–	
		24+24	–	1	1	1000–5000	−5000	53				–	Negative
				1	2	1000–5000	−5000	24				–	
				3	1	1250–5000	−5000	75				–	

		3 + 20	+	1	1	1000–5000			+1000 b	≥100 b	5.8 b	±	Negative
				1	2	1000–5000	−5000	98				−	
				2	2	625–5000			+625 m, b	76 m, b	6.5 m, 1.8 b	±	
				3	1	1250–5000	−5000	54				−	
		24 + 20	+	1	1	500–5000			+3500 b	80 b	3.1 b	±	Negative
				1	2	1000–5000	−5000	62				−	
				2	2	625–5000			+5000 b	60 b	2.1 b	±	
				3	1	1250–5000	−5000	93				−	
Diethylstilboestrol	56-53-1	3 + 21	−	1	2	7.5–40			+40	51	3.0	+	Positive
				2	1	0.5–10			+0.5	87	3.0	+	
		3 + 45	−	2	1	0.5–10			+0.5	≥100	11.8	+	Positive
		24 + 0	−	2	1	0.05–5			+0.05	≥100	11.4	+	Positive
		24 + 24	−	2	1	0.05–5			+0.05	≥100	30.3	+	Positive
		3 + 20	+	2	1	0.5–7			+7 m, + 3 b	49 m, 84 b	5.0 m, 9.0 b	+	Positive
		24 + 20	+	1	2	1.88–5			+5 m, +3.75 b	96 m, 87 b	3.9 m, 4.2 b	+	Positive
				2	1	0.05–1			+0.05 b	73 b	13.0 b	+	

[a] (−) No use of cytochalasin B; (+) use of cytochalasin B.

[b] Range of tested concentrations where genotoxicity was measured; p: precipitate at the highest concentration(s).

[c] (−) Negative, i.e. no significant increase in the number of micronucleated cells over the solvent control.

[d] Relative survival against the solvent control seen at the highest concentration based on cell counts in the absence of cytochalasin B or on percentages of binucleated cells when cytochalasin B was used.

[e] (+) Positive, i.e. significant increase in the number of micronucleated cells over the solvent control at $p < 0.05$ at at least one concentration; m: increase in the number of micronucleated mononucleated cells; b: increase in the number of micronucleated binucleated cells.

[f] Relative survival against the solvent control seen at the lowest positive concentration; m: in mononucleated cells, b: in binucleated cells where applicable.

[g] Maximal fold increase in micronucleated cells over the solvent control among the positive concentrations of the tested range, at relevant relative survivals (i.e. >40%); m: in mononucleated cells, b: in binucleated cells where applicable.

[h] (+) Positive: concentration-dependent increase in the number of micronucleated cells over the solvent control; (±) equivocal, i.e. significant increase seen at only one concentration or significant increases not concentration-dependent; (−) negative: no increase in the number of micronucleated cells over the solvent control at any concentration of the range.

[i] (+) Compound judged as positive in the treatment and recovery schedule (all the accepted assays were positive); (−) compound judged as negative in the treatment and recovery schedule (all the accepted assays were negative); (±) equivocal (controversial results between assays); if controversial results were obtained when the negative assay included too low concentrations or a too wide range of concentrations, only the positive assay was taken into account; on the opposite, if an equivocal response was not confirmed in another assay including an adequate range of concentrations, it was concluded as negative. The magnitude of the response was also considered.

[j] Only binucleated cells were evaluated.

seen only after the short treatment is consistent with this hypothesis. However this explanation alone may not account for all the micronucleated cells, as their levels were high in every case. More investigations would be necessary to determine if a part of these micronucleated mononucleated cells escaped from cytokinesis inhibition or passed mitosis without chromosome segregation.

3.4. Cytosine arabinoside

Cytosine arabinoside was found clearly positive in all treatment schedules, with and without cytochalasin B, with reproducible and concentration-dependent increases in the number of micronucleated cells, even at low cytotoxicity. In the presence of cytochalasin B, as seen with bleomycin, increases in micronuclei were observed in both binucleated as well as mononucleated cells.

3.5. Urethane

Urethane, tested up to 5000 μg/ml, produced low or no cytotoxicity and no increase in the number of micronucleated cells in the absence of cytochalasin B. It was clearly negative in all treatment schedules without cytochalasin B. When cytochalasin B was used, sporadic equivocal responses were found; but they were not concentration-related and not reproducible between the assays of a same laboratory and, in some instances, related to especially low spontaneous backgrounds. A third laboratory confirmed the negative results. Thus, urethane was also judged negative after the 3 and 24 h treatments in the presence of cytochalasin B.

3.6. Diethylstilboestrol

Only a few data remained available after evaluating the acceptability of assays, especially as no suitable cytotoxicity was achieved. This reflected the difficulty in finding concentrations sufficiently high to induce genotoxicity with an acceptable cytotoxicity. However, where adequate range of concentrations were obtained, clear concentration-dependent inductions of micronucleated cells were found, in all the treatment schedules, using cytochalasin B or not. In the presence of cytochalasin B, increases in micronuclei were observed in both binucleated as well as mononucleated cells.

4. Discussion

Mannitol was found negative in all treatment schedules, as expected with no cytotoxicity. Bleomycin was detected in all the treatment schedules. With cytochalasin B, an unexpected increase in the number of micronuclei in mononucleated cells would need more investigation to better understand its mechanism. The base analog cytosine arabinoside was also unambiguously found positive both in binucleated and mononucleated cells. Urethane was judged as negative in all the treatment schedules. These results must be considered in the light of published data and of the results obtained in other cell types in this collaborative study, which showed similar results [19]. This confirms previous published results reporting difficulties to detect this compound in vitro without an adequate specific metabolic activation. Indeed, there is some evidence that urethane might not be able to induce clastogenicity in vitro even in the presence of metabolic activation [22].

The aneugen diethylstilboestrol was found positive in all the treatment schedules, despite difficulties in finding a range of concentrations adequate to detect genotoxicity.

Among the different treatment schedules used, the results were comparable. No schedule appeared to give better results on this limited list of compounds. However, on a larger list of compounds, we may assume that the combination of a short and a long treatment would have been shown necessary for a full accurate evaluation of genotoxicity, as it was shown in other cell types in this collaborative study [19]. With or without cytochalasin B, for a given treatment schedule, the induction factors over the controls were of a same magnitude. Lastly, the evaluation of genotoxicity of the tested compounds in this study was concordant with published data [2,9,12,13,18].

In conclusion, the results of the present study show that CHO cells are suitable for detecting accurately genotoxic compounds of various types in the in vitro micronucleus test. All treatment-recovery schedules are suitable to detect genotoxic compounds. In any case, no genotoxic compound would have been missed with schedules including a short and a long treatment time, whatever the treatment was followed by a recovery period or not and whatever cytochalasin B was used or not. No differences were seen in the sensitivity or accuracy of the responses whether cytochalasin B was used or not.

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Appendix A. Individual data

Lab number	Assay number	CytB[a]	Schedule: treatment + recovery (h)	Solvent	Concentration (μg/ml)[b]	Micronucleated mononucleated cells[c]		Micronucleated binucleated cells[c]		Survival (%)	Number of cultures analysed	Others[d]	Acc.[e]	Pos.[f]
						Number per 1000 cells	Induction factor	Number per 1000 cells	Induction factor					
Mannitol														
Lab 1	Assay 1	–	3 + 21	Medium	0	22.0	1.0	na	na	nt	2	g	Y	NEG
					500	24.0	1.1	na	na	nt	2	g		
					1000	14.5	0.7	na	na	nt	2	g		
					1500	15.5	0.7	na	na	nt	2	g		
					2000	9.0	0.4	na	na	nt	2	g		
					2500	9.0	0.4	na	na	nt	2	g		
					3000	8.5	0.4	na	na	nt	2	g		
					3500	15.0	0.7	na	na	nt	2	g		
					4000	17.5	0.8	na	na	nt	2	g		
					4500	14.0	0.6	na	na	nt	2	g		
					5000	11.5	0.5	na	na	nt	2	g, p		
					MMC 0.2	188.0**	8.5	na	na	nt	2	g		
		–	3 + 45	Medium	0	23.5	1.0	na	na	100	2		Y	NEG
					100	13.0	0.6	na	na	70	2			
					200	14.0	0.6	na	na	67	2			
					700	nt	nd	na	na	49	2			
					800	nt	nd	na	na	50	2			
					900	17.0	0.7	na	na	50	2			
					1000	20.0	0.9	na	na	48	2	p		
					MMC 0.2	151.5**	6.4	na	na	90	2			
		–	24 + 0	Medium	0	20.5	1.0	na	na	100	2		Y	NEG
					100	25.0	1.2	na	na	47	2			
					200	18.5	0.9	na	na	48	2			
					700	nt	nt	na	na	48	2			
					800	nt	nt	na	na	51	2			
					900	21.0	1.0	na	na	48	2			
					1000	18.5	0.9	na	na	47	2	p		
					MMC 0.1	187.5**	9.1	na	na	51	2			
		–	24 + 24	Medium	0	32.5	1.0	na	na	nt	2	g	Y	NEG
					500	24.0	0.7	na	na	nt	2	g		
					1000	20.5	0.6	na	na	nt	2	g		
					3500	26.5	0.8	na	na	nt	2	g		
					4000	22.5	0.7	na	na	nt	2	g		
					4500	26.0	0.8	na	na	nt	2	g		
					5000	20.5	0.6	na	na	nt	2	g, p		
					MMC 0.1	111.0**	3.4	na	na	nt	2	g		