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Strategy for genotoxicity testing and stratification of genotoxicity test results—report on initial activities of the IWGT Expert Group

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During the past two decades, a number of national and international efforts have developed or refined the test methods and guidance on their strategic use to assess the potential genotoxicity of chemicals, including pharmaceuticals, pesticides, and industrial chemicals (selected papers and guidelines: [1–4]). Among the guidance documents agreed upon and used multinationally are the Organisation of Economic Cooperation and Development (OECD) protocol guidelines on individual tests and the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) test and strategy guidelines for pharmaceuticals [5–7]. In addition, recently, a Globally Harmonised System (GHS) for health hazard classification has been developed under the OECD auspices and is due to be formally ratified under the United Nations in December 2002 as part of a wider model scheme for hazard communication [8]. In the European Union (EU), there are various Directives and associated Annexes and Guidances that cover the regulatory requirements for mutagenicity testing and classification of dangerous substances [9–12] and the recently re-drafted Technical Guidance Document on Risk Assessment of New Substances, Existing Substances and Biocides [13]. Also recently, the United Kingdom Commit-

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tee on Mutagens (UKCOM) issued a strategic paper that incorporates many recent developments in test approaches and gives more weight than before on the analysis of numerical chromosomal aberrations [14].

To assist the continuous development of these national and international guidances, two major international conferences on genotoxicity test procedures under the International Workshop on Genotoxicity Tests (IWGT) initiative were held in Melbourne, Australia 1993 [15] and in Washington DC, United States 1999 [16]. These workshops invited recognised experts in genetic toxicity to work toward consensus approaches in genotoxicity testing and assessment. While an international consensus on test methods was the goal of these conferences, it was recognised that differences in legislation between countries often limit a harmonisation in their strategic use. In order to address the differences in national approaches, an expert group within the IWGT initiative was convened in Plymouth, England, in June 2002 to develop a process to provide guidance on a common strategy for genotoxicity testing and risk assessment. The authors of this summary paper comprise this IWGT strategy group, which includes experts from government, industry and academia. Many of these individuals have actively contributed to the development of the above mentioned strategies and guidances for genotoxicity testing (e.g. [17–21]).

The IWGT strategy group members noted that there is still a lack of common understanding of several issues that are considered important for the development of a consensus general strategy for genotoxicity testing and the interpretation of test results for risk assessment. While one initial goal of the group was to develop a possible classification scheme for genotoxicity, similar to the International Agency on Research on Cancer (IARC) classification of evidence for human carcinogenicity, it became evident during the discussions that the initial emphasis should be to formulate a common genotoxicity testing and assessment strategy. Therefore, the strategy group focused exclusively on these aspects during the Plymouth meeting.

The group agreed upon a number of principles, such as the need for an elementary data set that addresses the three major genetic endpoints, namely mutagenicity, chromosome breakage (clastogenicity), and aneugenicity. Table 1 summarises the consensus statements concerning genotoxicity testing and assessment. The strategy group understands that these consensus statements are basic elements that are to be considered independent from the area of use of test articles or human exposure/dose scenarios. Although the group consensus is that a minimum data set should always be

Table 1
Consensus statements as derived from discussions in the IWGT Strategy and Classification Working Group

Consensus 1: For hazard evaluation, data are needed from an elementary data set providing information on (1) gene mutations, (2) structural chromosome aberrations, and (3) numerical chromosome aberrations[a]
- The tests conducted to evaluate effects on these endpoints need to be properly conducted, i.e. according to existing guidelines, IWGT recommendations or best scientific practice
- Structure–activity analysis is a useful supplementary tool for assessment but is not essential[b]
- Investigations into biotransformation pathways (e.g. oxidative pathways and evidence for the formation of glutathione adducts), can provide useful initial information but are not essential for interpretation of results that have been obtained with the standard rat liver microsomal S9 mix[c]

Consensus 2: The three elementary genetic endpoints cannot currently be adequately covered with a single test system

Consensus 3: Useful information on the potential aneugenicity of a test chemical can be obtained by recording the incidence of polyploidy and/or mitotic index in the in vitro cytogenetic test. A higher level of confidence in the detection of aneuploidy can be obtained by studying the induction of micronuclei and/or non-disjunction (in vitro or in vivo)

Consensus 4: In some instances, in vivo studies are mandated or are part of the testing program (e.g. for pharmaceuticals). If in such cases an aneuploidy endpoint (e.g. as detected with the in vitro micronucleus test or the assessment of polyploidy and mitotic index in the in vitro cytogenetic assay) has not been studied in vitro, it should be studied in vivo

[a] It is recognised that at this time, existing regulations/guidelines/guidances/regulatory practices allow preliminary assessments on the basis of a data set that may not include all of the components of the elementary data set.

[b] Most structure–activity methods mainly address potential direct DNA reactivity.

[c] A preliminary characterisation of the metabolism of the test compound in the in vitro genotoxicity test systems is useful.

available, it is acknowledged that in practice there are cases with such a low exposure potential that judgements on the basis of more limited data may be justified.

Starting from these consensus statements, the various regulatory strategies, their scientific assumptions and their potential shortcomings were discussed. In view of the limited time available at this first meeting, it was recognised that a consensus on most major issues could not be achieved. It was therefore unanimously decided to identify major open questions and needs that would be addressed subsequently by independent working groups. These working groups would be formed after the meeting to work toward a consensus on the issues identified below.

(1) *Agents mutagenic in vivo but non-mutagenic in vitro.* Several current guidance approaches, in particular for the testing of pharmaceuticals, stipulate that an in vivo test should normally be part of an initial test battery for genotoxicity. This approach was implemented because of examples of agents that are mutagenic in vivo but not reliably detected as mutagenic in standard in vitro tests. These examples include procarbazine, urethane, benzene and hydroquinone as referred to in the ICH S2B guidance [7,15]. However, for most of such compounds, positive results are available from in vitro tests when the tests are adapted to the specific characteristics of the test compound. Nevertheless, the attendees felt that there were additional relevant unpublished examples, which need to come to the attention of the scientific community, if possible. The group felt that the occurrence of agents that are uniquely mutagenic in vivo was relatively rare, but that a survey should be made of unpublished evidence to provide substantive data on this point. It was suggested that a questionnaire should be drafted and an effort made to identify further examples of such agents. This questionnaire should address the following questions:
   - Are there (further) examples of uniquely in vivo positives that would justify the use of an in vivo test in the initial battery of tests?
   - Are there convincing examples of differences between in vitro and in vivo metabolism that would account for such a data set?
   - Are there examples of inappropriately low exposure to relevant metabolites in vitro?

(2) *Rationale for in vivo testing of agents that are negative after thorough in vitro testing.* In certain cases, there may be reasons to conduct in vivo testing even after in vitro tests have been conducted to the best standards and scientific knowledge and have failed to identify mutagenic activity. The following three questions are designed to identify follow-up scenarios if data from standard in vitro tests are negative, but other information, such as studies on metabolism, indicate that in vivo tests would be helpful.
   - Which endpoints/tissues should be studied in vivo to confirm/investigate negative results in vitro?
   - What strategy is appropriate if structure–activity data suggest that the conventional in vitro assays may yield irrelevant results?
   - What evidence indicates a need to conduct follow-up tests in vivo (e.g. are there examples for in vivo metabolism known to generate potentially active intermediates, which are not produced by the in vitro metabolising system)?

(3) *Follow-up testing of tumourigenic agents not positive in the "standard" genotoxicity test battery.* It is acknowledged that a carcinogenicity test in rodents can yield evidence for a tumourigenic response of a compound that is negative in a genotoxicity test battery in vitro and in vivo. The ICH guidance S2B [7] stipulates that such compounds shall be investigated further in supplemental genotoxicity tests, if rodent tumourigenicity is not clearly based on a non-genotoxic mechanism. Cases for which there is no clear information about the mechanism of tumourigenesis occur often in rodent life-time bioassays. Hence, examples of compounds that induce tumours at specific sites in rodent carcinogenicity studies, but which are clearly negative in vitro and in vivo in the elemental data set, and which have been subsequently tested in additional genotoxicity tests could be important for evolving a more appropriate genotoxicity testing strategy. A subgroup will examine such cases, the scientific rationales that were applied, and the types of tests that were conducted. It is understood that these examples will most likely be available in

the pharmaceutical area and will have to include the recently developed mouse transgenic carcinogenicity assays, in particular those with an activated human ras gene and an inactivated p53 tumor suppressor gene allele.

(4) *Metabolic considerations.* Genotoxicity testing relies heavily on rodent metabolism systems for in vitro tests (normally aroclor- or phenobarbital-plus 5,6-benzoflavone-induced rat liver S9) and rodents are usually used for in vivo investigations. Also, carcinogenicity testing is usually performed with rodents (rats and/or mice). However, there are examples of major human metabolites that are not presented under the conditions in vitro or in rodents in vivo. Under these circumstances, additional testing may be indicated. The following questions are related to this issue.
- When is a change in an exogenous metabolic system in vitro indicated?
- Which animal species in vivo should be selected?
- When shall the human metabolite be isolated or synthesised and tested separately?

(5) *Dose–response considerations.* Recently, evidence for (i) non-linear dose–response relationships, (ii) responses only at high or cytotoxic doses, and (iii) mechanisms of genotoxicity that may involve a threshold have been important topics of discussion in the field of genetic toxicology. These aspects have not been treated with consistency in the regulatory evaluation of genotoxicity. In this context, the following questions have been raised during the group discussions as relevant for a more consistent approach towards the interpretation of genotoxicity test results:
- When do positive results in bacterial or in vitro mammalian cell mutation tests indicate (or not indicate) a human hazard?
- When can a negative mammalian cell gene mutation test, e.g. a mouse lymphoma tk assay, overrule a positive bacterial assay result?
- Are there examples of positives resulting from bacterial-specific metabolism/mechanisms, which may be not relevant for the mammalian organism and what are the appropriate measures to show this?
- How can positive results from in vitro micronucleus assays be interpreted?
  1. Is the test agent an aneugen and does this imply the existence of a threshold?
  2. Is the test agent a clastogen and how can sufficient evidence for a non-linear dose–response or non-relevance be provided?
- Can reliable criteria be developed to identify when positive genotoxicity results are either not relevant, follow a non-linear dose–effect relationship or demonstrate a threshold, in particular for chromosomal aberration or mouse lymphoma tk tests in vitro and for genotoxicity assays in vivo?

(6) *Risk assessment versus hazard identification.* There is considerable debate over how to conduct risk assessment based on genotoxicity test results. Traditionally, risk assessment considers in vivo dose–response data, information on the mechanism of action, and information on human exposure. Since genotoxicity testing employs several in vitro approaches, there is a need to assess how in vitro data can contribute to a risk assessment. A fundamental question is whether or how reliably information on a genotoxic potency can derived from in vitro test data and how this information can be used quantitatively in risk evaluation?

These six general areas of discussion were considered to be the most critical for the strategic use of test systems and interpretation of genotoxicity test results. Ultimately, the goal of addressing the issues outlined above is to develop consistent strategies for genotoxicity testing and follow-up that allow an assessment of potential for human risk. For each of the above tasks, potential work group leaders have been identified and a procedure of regular meetings or conferences has been developed.

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Summary of major conclusions

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1. Introduction

Comprehensive summaries of the outcome of each working group are given in the individual workgroup reports. The following outline summarises the main points that either differ from existing published recommendations (as in the case of mouse lymphoma test) or are key features to be considered in the development of new guidelines.

2. Protocol design

Historically, there has been a tendency to recommend protocols that are as extensive as possible in the hopes of not failing to identify a genotoxic chemical. Based on previous experience [1,2] and the large databases that are now available for all of the assays evaluated in this workshop, it is clear that no assay, however extensive the protocol, can detect all genotoxic chemicals. It is also clear that safety assessments of chemicals regarding genotoxicity are generally based on a combination of tests, i.e. a test battery. Therefore, the working groups dealing with test methods were asked to define the basic features of the protocol that are essential for the detection of the majority of genotoxins. They were then encouraged to identify special cases, i.e. compounds or classes of compounds, for which specific protocol adaptations might be needed.

3. Mouse Lymphoma Assay

This group has met a number of times since the Washington workshop [3], and had achieved a number of consensus agreements prior to the Plymouth workshop, e.g. confirming the usefulness of the 24-h treatment protocol that was originally introduced in the ICH guidance on "A standard battery for genotoxicity testing of pharmaceuticals" [4]. The group also confirmed that if test cultures give clearly negative results, then an acceptable study would be comprised of a single test with 3–4 h exposures without and with S9 and a 24 h exposure without S9.

The main objectives of the Plymouth workshop were to establish criteria for assay acceptability and approaches to data evaluation. Data sets from 400 experiments carried out in 10 different laboratories were reviewed. Based on these data, the group made recommendations for acceptable ranges for spontaneous mutant frequency, cloning efficiency and suspension growth for both agar and microwell methods.

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The reliability and quality of a performed test can often be determined from the positive control response. Therefore, it was deemed desirable to provide recommendations on appropriate controls and expected responses. However, it proved quite difficult to agree on recommendations for positive controls. A wide variety of positive control chemicals have been used at many different doses. The working group proposed to select (what is hoped will be) universally acceptable positive controls, and to define their properties.

For statistical evaluation, 398 data sets and 29 different statistical methods were reviewed. A variety of methods were found to be acceptable for analysis of dose–response, but no single statistical method could be recommended to define results as positive or negative. The group has suggested the best approach is to determine a "global evaluation factor" above which an induced mutant frequency would need to rise before statistical methods were applied. A decision on the result of the study would be made on the basis of both biological and statistical approaches. This approach will be further evaluated with the 398 data sets already available, and by individual laboratories with additional individual data sets.

4. In Vivo Transgenic Mutation Assays

The recommendations for treatment and sampling times that were based on theory at the Washington workshop were reviewed. From analysis of a database of 155 compounds, it was agreed that treatment for 28 days with sampling 3 days later would detect most mutagens. However, it was also concluded that this recommendation could be modified by reducing or increasing the number of treatments, or the length of the treatment period, when scientifically justified. It was the consensus that male animals alone are usually sufficient, and, unless existing information suggests otherwise, at least one rapidly proliferating and one slowly proliferating tissue should be sampled.

Sequencing of mutants was not considered necessary for a clearly negative or clearly positive result. However, sequencing data are useful to identify and correct for "jackpot" mutations, to investigate high variability in controls, and to resolve equivocal results.

5. In Vitro Micronucleus Test

It was agreed that cell proliferation should be demonstrated in both control and treated cells. Cytochalasin B can be used with cell lines (optional), but should be used for human lymphocytes and to measure non-disjunction. In cell lines without cytochalasin B, demonstrating a greater number of cells at harvest than at the start of treatment will verify proliferation.

For an acceptable study, the consensus was that at least three dose levels should be used, at intervals no more than $\sqrt{10}$. The highest concentration should produce at least 60% toxicity; this may be needed to detect some aneugens.

Detailed treatment and sampling protocols have been recommended both for cell lines (with and without cytochalasin B) and for human lymphocytes. The recommended methods differ slightly from each other (because of the need to treat exponentially growing cells) but, because of the need to reach 60% toxicity, also differ from the recommended protocol for the in vitro chromosomal aberration test [5]. The debate has been extensive in this area and the situation will need to be reviewed in the future. It is also recommended that 1000 target cells per culture are scored for micronuclei, with parallel scoring of 1000 mononucleated cells in those cultures with cytochalasin B where the target cells will be 1000 binucleates. It is hoped that the recommendations on this assay would serve as a useful basis to place the in vitro micronucleus test into the regulatory arena of accepted GLP tests according to OECD, ICH and other product testing guidances.

6. Genetic and Molecular Analysis of Tumour and Non-tumour Tissue in p53-deficient and rasH2 Transgenic Tumour Models

The working group reviewed the experimental data from the p53 and rasH2 tumour models on about 100 chemicals so far tested, and also reviewed the data related to genetic drift in the Tg.AC model. The strengths and weaknesses of the various models were also discussed. Although experience with the Tg.AC model illustrates the need for vigilance, the working group agreed that the p53 and rasH2 models have been appropriately genetically characterised by their creators

and that the genetic characteristics are now generally routinely characterised by the suppliers in the maintenance of foundation and breeding colonies. There was no consensus on whether further characterisation by the end user was needed, but prudent practice would indicate that tissue (before and after treatment) should be saved in case needed for subsequent analysis.

In tumours, the working group noted that analysis of mutational spectra in critical activating codons of the c-Ha-ras gene, or loss of heterozygosity of the p53 gene, would be unlikely to define a study result as positive or negative (so many tumours of large size would be needed for the analysis, that a positive outcome would already have been obvious), but it was agreed that in certain circumstances further molecular characterisation of induced tumours would provide useful information in elucidating the mode of action of a tumourigenic chemical. Available experimental approaches to characterise genetic changes in tumours that can provide significant evidence of a direct genotoxic mechanism of action for some carcinogens were discussed. It was noted, however, that the absence of any positive findings in such molecular analyses cannot provide evidence for the absence of a direct genotoxic mechanism. Hence, although useful for carcinogenicity testing, it seems presently unrealistic to expect that the (genotoxic) mechanism of action, or the absence of such a mechanism, can be reliably assessed in these models.

7. Classification of Genotoxins and Strategy for Risk Assessment

Earlier IWGT workshops have focused on recommendations for the best ways to conduct tests. This new workgroup addressed ways in which the tests can be used strategically. This is a major undertaking, and it is recognised that this will be an extended process. Prior to the meeting it was envisaged that a classification scheme for hazard identification would be a good place to start, as this should lead to definition of the minimum tests needed to achieve a particular classification. This should then lead to a strategic approach to testing which could be applied similarly to industrial chemicals, pharmaceuticals, agrochemicals, food additives, etc. However, it soon became apparent that there was no consensus for a classification scheme. There was serious concern that classification would lead to "labels" that could have serious commercial consequences, and this was not intended by this science-focused working group.

The working group therefore decided to focus on the development of a universally acceptable, step-wise process for genotoxicity hazard evaluation and risk assessment. In particular, there is a need to encompass the different approaches to hazard evaluation and to remove the inconsistencies in regulatory interpretation.

The group agreed to a mission statement and some consensus statements on hazard evaluation, related mainly to the elementary data set on which hazard evaluation could be based. The group defined the key issues that need to be addressed in order to stratify the "meaning" of genotoxicity test results relative to each other, and has formed working subgroups to evaluate these issues and to report their progress at the next IWGT workshop planned in conjunction with the 2005 ICEM in San Francisco.

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The effect of aging on the results of the rat micronucleus assay

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We conducted the micronucleus assay in 3-, 5-, 7-, 9-, 11- and 13-week-old male rats to determine whether the results varied with age. We administered cyclophosphamide orally at 0 (vehicle), 5, 10 or 20 mg/kg, twice, 24 h apart, to five rats per dosage group and collected bone marrow and peripheral blood 24 h after the second treatment. We observed an age-related decrease in micronucleus-inducing activity in both polychromatic erythrocytes (bone marrow) and reticulocytes (peripheral blood), which we attributed to an age-related decrease in hematopoiesis. In spite of the age-related decrease in sensitivity to the model chemical studied here, all age groups tested showed positive responses. We concluded that the rat is suitable for the micronucleus assay regardless of age.

Introduction

The mouse is conventionally used for the micronucleus assay while the rat is usually used for general toxicologic and toxicokinetic studies. If micronucleus induction can be evaluated in the same rats that are used for general toxicology studies, a lot of information can be gathered concomitantly. We explored the possibility of incorporating micronucleus assays into 28 day repeat dose general toxicology studies, where the effect of aging could be important, using the same animals (Hamada *et al.*, 2001). The Collaborative Study Group for the Micronucleus Test (CSGMT), a working group of the Mammalian Mutagenicity Study Group (MMS), which is a subgroup of the Japanese Environmental Mutagen Society (JEMS), previously evaluated the effects of aging on the micronucleus assay in mice (CSGMT, 1995a; Sato *et al.*, 1995; Hamada *et al.*, 1996; Higashikuni and Sutou, 1996). In the present study, we investigated the aging effects in rats using cyclophosphamide (CP) as the model chemical.

Materials and methods

Chemicals

Cyclophosphamide (CAS no. 50-18-0, lot no. 9014), obtained from Shionogi & Co. Ltd (Osaka, Japan), was dissolved in distilled water immediately before use.

Animals

Male Crj:CD(SD)IGS rats 3, 5, 7, 9, 11 and 13 weeks old were purchased from Charles River Japan Inc. They were given commercial pellets and tap water *ad libitum* throughout the acclimatization and experimental periods and were subjected to a 12 h light/dark cycle.

Micronucleus assay protocols

To perform the micronucleus assay using bone marrow and peripheral blood simultaneously in the same animal, a double dosing regimen (CSGMT, 1990, 1995b) was used. CP dissolved in distilled water at 0 (vehicle), 5, 10 or 20 mg/kg was administered orally twice, 24 h apart, to five rats per dosage group. Both peripheral blood and bone marrow were collected 24 h after the second treatment. Acridine orange staining (Hayashi *et al.*, 1983) was used for analysis. Micronucleated polychromatic erythrocyte (MNPCE), micronucleated normochromatic erythrocyte (MNNCE) and micronucleated reticulocyte (MNRET) frequencies were recorded based on the observation of 2000 polychromatic erythrocytes (PCEs), 2000 normochromatic erythrocytes (NCEs) or 2000 reticulocytes (RETs). Cytotoxicity was evaluated based on the observation of 300 bone marrow erythrocytes or 2000 peripheral blood reticulocytes.

Statistical analysis

To compare the responses of each dosage group with those of the concurrent control group, we used Kastenbaum and Bowman's tables (Kastenbaum and Bowman, 1970) for the frequency of MNPCEs, MNRETs and MNNCEs and the *t*-test for the frequency of PCEs and RETs.

Results

Table I shows the results of both assays. In the bone marrow assay, the MNPCE frequency increased significantly in the treated animals in all dosage and age groups. The magnitude of this increase decreased with increasing age in the 10 and 20 mg/kg/day groups. There were no significant increases in MNNCE frequency in any group treated at 5 mg/kg/day. The PCE frequency was highest in the 3-week-old age group and decreased significantly at all doses in the 9-, 11- and 13-week-old groups. The decreases were age-related.

In the peripheral blood micronucleus assay, the MNRET frequency was increased significantly in all dosage groups by CP at 5, 7, 11 and 13 weeks of age. The magnitude of the increases decreased in an age-related manner within the 5, 10 and 20 mg/kg/day groups up to 7–9 weeks of age, but not in the vehicle control groups. There was no age- or dose-dependent increase in the frequencies of MNNCE. The RET frequency decreased significantly in treated animals at all dosages in the 3-, 7- and 9-week-old groups and at 20 mg/kg/day in all age groups, relative to the 3-week-old group. An age-related decrease was seen in all dosage groups.

Discussion

There was a tendency towards an age-related decrease in micronucleus assay sensitivity to CP in the rat bone marrow assay. The sensitivity of the assay may depend on the metabolic activity of cytochrome P450 2C enzyme (CYP2C), which metabolizes CP to a genotoxic intermediate (Clarke and Waxman, 1989), but this needs further evaluation. Yajima *et al.* (1993a–c) and Handa and Yajima (1995) speculated that the increased MNPCE and MNRET frequencies are the result of errors that occur in the processes of denucleation, differentiation and repair of genetic damage. They also suggested a close relationship between erythropoiesis and micronucleus

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Table I. Micronucleus induction in male SD rats treated with cyclophosphamide ($n = 5$)

Age (weeks)	Dose (mg/kg)	Bone marrow			Peripheral blood		
		MNPCE (%)	MNNCE (%)	PCE (%)	MNRET (%)	MNNCE (%)	RET (%)
3	0	0.12 ± 0.06	0.01 ± 0.02	85.72 ± 5.10	0.14 ± 0.06	0.03 ± 0.05	19.58 ± 1.64
	5	0.71 ± 0.23[a]	0.02 ± 0.03	85.84 ± 5.77	0.36 ± 0.23	0.01 ± 0.02	16.62 ± 0.54[a]
	10	2.27 ± 0.37[a]	0.08 ± 0.06[b]	85.14 ± 6.20	1.26 ± 0.32[a]	0.04 ± 0.04	17.96 ± 1.65
	20	5.15 ± 1.00[a]	0.20 ± 0.13[a]	69.28 ± 9.41[b]	1.47 ± 0.52[a]	0.04 ± 0.07	10.60 ± 2.24[a]
5	0	0.15 ± 0.06	0.02 ± 0.03	84.22 ± 3.09	0.04 ± 0.02	0.00 ± 0.00	11.80 ± 0.78
	5	0.95 ± 0.17[a]	0.02 ± 0.03	81.78 ± 2.16	0.48 ± 0.16[a]	0.01 ± 0.02	11.02 ± 1.55
	10	1.55 ± 0.15[a]	0.07 ± 0.06	76.10 ± 5.41[b]	1.01 ± 0.32[a]	0.02 ± 0.03	9.94 ± 0.81[a]
	20	4.66 ± 0.94[a]	0.19 ± 0.07[a]	55.72 ± 4.98[a]	1.29 ± 0.34[a]	0.01 ± 0.02	5.96 ± 0.65[a]
7	0	0.13 ± 0.06	0.02 ± 0.03	73.18 ± 5.01	0.03 ± 0.03	0.00 ± 0.00	6.18 ± 0.63
	5	0.70 ± 0.15[a]	0.01 ± 0.02	72.16 ± 5.47	0.29 ± 0.27[b]	0.00 ± 0.00	5.44 ± 0.46[b]
	10	1.44 ± 0.32[a]	0.04 ± 0.04	69.98 ± 2.53	0.77 ± 0.18[a]	0.01 ± 0.02	4.02 ± 0.58[a]
	20	4.34 ± 0.72[a]	0.14 ± 0.07[a]	49.48 ± 6.47[a]	1.05 ± 0.41[a]	0.02 ± 0.03	3.02 ± 0.78[a]
9	0	0.11 ± 0.04	0.02 ± 0.03	77.88 ± 4.06	0.06 ± 0.06	0.01 ± 0.02	4.08 ± 0.49
	5	0.62 ± 0.11[a]	0.02 ± 0.03	68.94 ± 2.13[a]	0.17 ± 0.07	0.02 ± 0.03	2.98 ± 0.81[b]
	10	1.43 ± 0.14[a]	0.04 ± 0.04	65.52 ± 3.77[a]	0.57 ± 0.16[a]	0.00 ± 0.00	2.24 ± 0.65[a]
	20	3.61 ± 0.68[a]	0.17 ± 0.08[a]	37.24 ± 5.72[a]	1.24 ± 0.48[a]	0.01 ± 0.02	0.96 ± 0.25[a]
11	0	0.08 ± 0.03	0.01 ± 0.02	67.80 ± 2.00	0.02 ± 0.03	0.01 ± 0.02	1.72 ± 0.46
	5	0.68 ± 0.15[a]	0.01 ± 0.02	55.40 ± 4.81[a]	0.24 ± 0.06[b]	0.00 ± 0.00	1.86 ± 0.19
	10	1.21 ± 0.23[a]	0.06 ± 0.04	36.18 ± 4.53[a]	0.66 ± 0.27[a]	0.01 ± 0.02	1.22 ± 0.31[b]
	20	3.13 ± 0.35[a]	0.08 ± 0.03[b]	28.42 ± 9.43[a]	1.20 ± 0.24[a]	0.00 ± 0.00	0.70 ± 0.28[a]
13	0	0.15 ± 0.06	0.02 ± 0.05	62.12 ± 4.15	0.03 ± 0.03	0.00 ± 0.00	1.72 ± 0.24
	5	0.54 ± 0.11[a]	0.04 ± 0.04	51.88 ± 3.33[a]	0.25 ± 0.11[a]	0.01 ± 0.02	1.86 ± 0.27
	10	0.89 ± 0.08[a]	0.05 ± 0.05	40.64 ± 7.05[a]	0.49 ± 0.18[a]	0.00 ± 0.00	1.08 ± 0.30[a]
	20	2.94 ± 0.87[a]	0.20 ± 0.04[a]	29.58 ± 4.58[a]	1.34 ± 0.45[a]	0.01 ± 0.02	0.74 ± 0.26[a]

Values shown are means ± SD. MNNCE, micronucleated normochromatic erythrocytes; MNPCE, micronucleated PCE; MNRET, micronucleated RET; PCE, polychromatic erythrocytes; RET, reticulocytes.
[a] $P < 0.01$ compared with the results of the vehicle control group in each age.
[b] $P < 0.05$ compared with the results of the vehicle control group in each age.

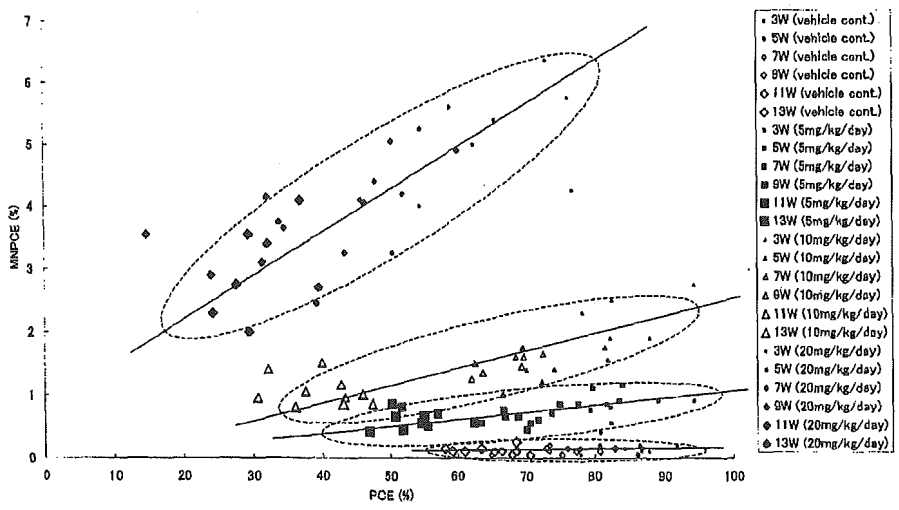

Fig. 1. Relationship of MNPCE and PCE frequencies in bone marrow of male SD rats following oral administration of CP.

induction. In the present study, there was a close relationship between PCE and MNPCE frequencies at the same CP dose (Figure 1). These results suggested that the young animals, which had a high level of erythropoietic activity, tended to show high micronucleus induction. We confirmed an age-related reduction in sensitivity to CP, although all dosages induced MNPCE significantly. At 5 mg/kg/day, CP did not significantly increase MNNCE frequency in any age group. These results might suggest that the micronucleus-inducing activity of compounds could be evaluated in bone marrow cells regardless of age of rat if MNPCEs, but not MNNCEs, were scored.

The results of the peripheral blood assay were similar to those of the bone marrow assay. The micronucleus-inducing activity of compounds could be evaluated regardless of age if MNRETs, but not MNNCEs, were scored. A good correlation between RET and MNRET frequencies was seen within the same CP dosages, but the peripheral blood data varied more widely than the bone marrow data (Figure 2), probably because some circulating micronucleated erythrocytes were destroyed in the spleen (Schlegel and Macgregor, 1982, 1984; Schlegel *et al.*, 1986; Hayashi *et al.*, 1992), but that needs further evaluation.

The effect of aging on the micronucleus assay was not so

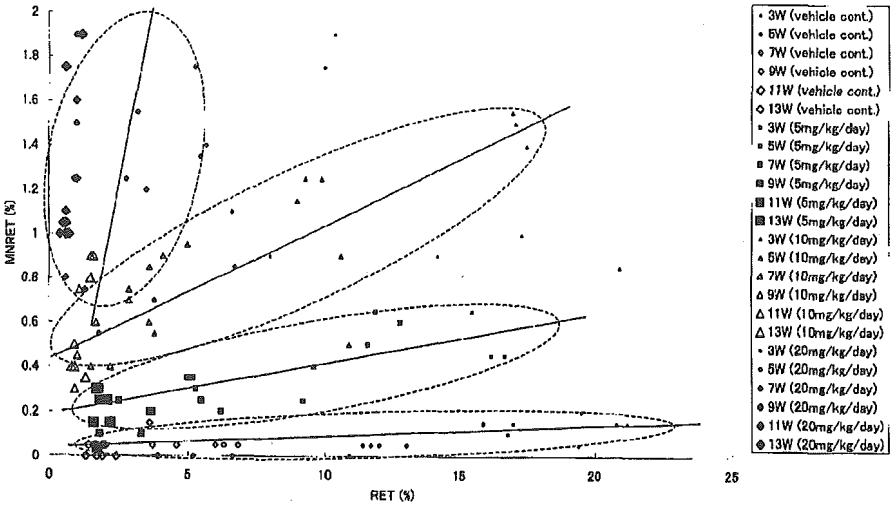

Fig. 2. Relationship of MNRET and RET frequencies in peripheral blood of male SD rats following oral administration of CP.

clear in the mouse (Hamada *et al.*, 1996) as in this study of the rat. The lowering of erythropoiesis with age in the mouse was less (Hamada *et al.*, 1999) than in the rat, which was assumed to be the primary cause of the small effect of aging in the mouse micronucleus assay.

In summary, these results suggest that the effect of aging in the micronucleus assay depends mainly on age-related changes in erythropoietic function and the chemical induction of micronuclei in bone marrow cells can be evaluated by scoring MNPCEs in the bone marrow or MNRETs in the peripheral blood in the rat regardless of age, with marginal age-dependent differences in sensitivity.

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Sex differences in the chemical induction of micronuclei in the rat

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Summary

The micronucleus assay was conducted with 7 chemicals (2-acetylaminofluoren [2-AAF], 1-β-D-arabinofuranosylcytosine [Ara-C], colchicine, cyclophosphamide [CP], methyl methanesulfonate [MMS], potassium bromate [$KBrO_3$], urethane) in male and female rats to determine whether the results varied with sex. Each chemical was administered twice orally, 24 h apart, to 5 rats in each of 3 dosage groups and collected bone marrow and peripheral blood 24 h later. Sex differences were observed in micronucleus induction in both polychromatic erythrocytes (bone marrow) and reticulocytes (peripheral blood), which we attributed to a sex difference in hematopoiesis. In spite of those differences, both sexes showed positive responses. We concluded that the rat is suitable for the micronucleus assay regardless of sex.

Keywords : micronucleus, rat, sex, hematopoiesis, CSGMT

Introduction

The mouse is conventionally used for the micronucleus assay while the rat is usually used for general toxicologic and toxicokinetic studies. If micronucleus induction could be evaluated in the same animals that are used in 28-day repeat-dose general toxicology studies, more data could be gathered concomitantly. The Collaborative Study Group for the Micronucleus Test (CSGMT) has been exploring that possibility (Hamada et al., 2001a). CSGMT is a working group of the Mammalian Mutagenicity Study Group (MMS) which is a subgroup of the Japanese Environmental Mutagen Society (JEMS). CSGMT previously evaluated several factors that could affect the results of the micronucleus assay in mice and is now conducting similar studies in rats. We have so far evaluated the effects of strain (Hamada et al., 2001b) and aging (Hamada et al., 2003). In the present study, we used 7 chemicals to investigate the effects of sex.

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Materials and Methods

Chemicals

Seven chemicals (Table 1) were selected to cover some classes of mutagens, such as nucleoside analog, alkylating agent, mitotic inhibitors, and so on. These chemicals are positive in both rat (male) and mouse (male and female) short-term micronucleus assays (Hayashi et al., 1989 ; CSGMT, 1990, 1992 ; Morita et al., 1997 ; Wakata et al., 1998), and 2-AAF is reported to show different responses between male mice and female mice (CSGMT, 1986).

Animals

Six-week-old male and female F344/DuCrj rats were purchased from Charles River Japan Inc. and acclimated for one week. They were given commercial pellets and tap water ad libitum throughout the acclimatization and experimental periods and were subjected to a 12-hour light/dark cycle. They were 7 weeks old at the start of the study.

Table 1 Chemicals used in micronucleus assays

Chemicals (abbreviation)	CAS no	Classification	Dose (mg/kg/day)
2-Acetylaminofluorene (2-AAF)	53-96-3	Aromatic amine	100, 200, 400
1-β-D-Arabinofuranosylcytosine (Ara-C)	147-94-4	Nucleoside analog	160, 320, 640
Colchicine	64-86-8	Spindle poison	2.5, 5, 10
Cyclophosphamide (CP)	6055-19-2	Bis compound	5, 10, 20
Methyl methanesulfonate (MMS)	66-27-3	Alkyl sulfonate	10, 20, 40
Potassium bromate ($KBrO_3$)	7758-01-2	Oxidant	80, 160, 320
Urethane	51-79-6	Carbamate	100, 200, 400

Table 2 Micronuclei induced by test chemicals in male and female rats

Chemicals	Sex	Dose (mg/kg)	Number of rats	Bone marrow			Peripheral blood			Sex difference	
				MNPCE(%)	MNNCE(%)	PCE(%)	MNRET(%)	MNNCE(%)	RET(%)	MNPCE	MNRET
Control	♂	0	5	0.04 ± 0.04	0.01 ± 0.02	69.18 ± 9.55	0.04 ± 0.04	0.01 ± 0.02	3.74 ± 0.44	ns	ns
	♀	0	5	0.03 ± 0.03	0.01 ± 0.02	64.30 ± 4.94	0.03 ± 0.03	0.01 ± 0.02	2.42 ± 0.19		
2-AAF	♂	100	5	0.18 ± 0.10**	0.02 ± 0.03	55.84 ± 9.15	0.06 ± 0.06	0.00 ± 0.00	3.00 ± 0.14*	ns	ns
		200	5	0.13 ± 0.05**	0.01 ± 0.02	53.22 ± 5.30*	0.05 ± 0.04	0.01 ± 0.02	2.94 ± 0.43*		
		400	5	0.30 ± 0.00**	0.00 ± 0.00	46.02 ± 8.18**	0.04 ± 0.04	0.01 ± 0.02	3.10 ± 0.20*		
	♀	100	5	0.09 ± 0.04	0.01 ± 0.02	47.84 ± 4.73*	0.07 ± 0.03	0.00 ± 0.00	2.06 ± 0.32		
		200	5	0.10 ± 0.04*	0.01 ± 0.02	40.66 ± 7.46**	0.05 ± 0.04	0.00 ± 0.00	1.84 ± 0.18*		
		400	5	0.30 ± 0.04**	0.00 ± 0.00	43.40 ± 5.47**	0.05 ± 0.04	0.00 ± 0.00	2.10 ± 0.42		
Ara-C	♂	160	5	0.91 ± 0.09**	0.03 ± 0.03	54.22 ± 13.90	0.07 ± 0.03	0.00 ± 0.00	2.72 ± 0.29*	M**	ns
		320	5	1.54 ± 0.36**	0.09 ± 0.06*	34.62 ± 8.76**	0.18 ± 0.08**	0.00 ± 0.00	1.60 ± 0.30**		
		640	5	1.55 ± 0.30**	0.07 ± 0.03*	18.38 ± 6.65**	0.17 ± 0.07**	0.00 ± 0.00	1.08 ± 0.20**		
	♀	160	5	0.54 ± 0.12**	0.02 ± 0.03	38.16 ± 2.31**	0.07 ± 0.05	0.00 ± 0.00	2.06 ± 0.18		
		320	5	0.70 ± 0.20**	0.02 ± 0.03	15.34 ± 2.92**	0.13 ± 0.03**	0.00 ± 0.00	0.90 ± 0.16**		
		640	5	1.35 ± 0.20**	0.07 ± 0.03*	10.72 ± 4.46**	0.18 ± 0.03**	0.01 ± 0.02	0.92 ± 0.08**		
Cholchicine	♂	2.5	5	0.08 ± 0.03	0.01 ± 0.02	57.62 ± 7.71	0.05 ± 0.05	0.00 ± 0.00	3.64 ± 0.11	ns	ns
		5	5	0.18 ± 0.12**	0.00 ± 0.00	59.48 ± 4.05	0.04 ± 0.02	0.00 ± 0.00	3.32 ± 0.59		
		10	5	0.31 ± 0.12**	0.01 ± 0.02	49.62 ± 6.50*	0.09 ± 0.04	0.02 ± 0.03	2.62 ± 0.41*		
	♀	2.5	5	0.04 ± 0.04	0.00 ± 0.00	49.76 ± 7.27*	0.05 ± 0.04	0.00 ± 0.00	2.26 ± 0.09		
		5	5	0.12 ± 0.05**	0.00 ± 0.00	52.40 ± 7.26*	0.05 ± 0.04	0.00 ± 0.00	1.96 ± 0.31*		
		10	5	0.31 ± 0.15**	0.00 ± 0.00	49.76 ± 4.97*	0.07 ± 0.03	0.00 ± 0.00	1.70 ± 0.21*		
CP	♂	5	5	1.10 ± 0.29**	0.08 ± 0.06*	48.12 ± 10.58*	0.20 ± 0.11**	0.01 ± 0.02	3.36 ± 0.91	M**	ns
		10	5	2.63 ± 0.48**	0.15 ± 0.04**	35.10 ± 6.91**	0.25 ± 0.06**	0.00 ± 0.00	2.38 ± 0.53**		
		20	5	2.95 ± 0.63**	0.15 ± 0.06**	18.42 ± 7.03**	0.29 ± 0.11**	0.01 ± 0.02	1.02 ± 0.31**		
	♀	5	5	0.55 ± 0.25**	0.02 ± 0.03	38.40 ± 14.61**	0.14 ± 0.08**	0.01 ± 0.02	3.02 ± 0.77		
		10	5	1.58 ± 0.08**	0.09 ± 0.07*	25.44 ± 4.50**	0.21 ± 0.04**	0.01 ± 0.02	1.74 ± 0.91*		
		20	5	0.96 ± 0.44**	0.03 ± 0.05	23.58 ± 5.12**	0.21 ± 0.07**	0.02 ± 0.03	1.04 ± 0.49**		

Data shown in the table are mean ± SD. * $P < 0.05$, ** $P < 0.01$, compared with the results of vehicle control group in each sex.
PCE : polychromatic erythrocytes, RET : reticulocytes, MNPCE : micronucleated PCE, MNRET : micronucleated RET, MNNCE : micronucleated normochromatic erythrocytes. Sex difference : ns ; not significant, M** ; males are significantly more sensitive than females ($P < 0.01$)

Micronucleus assay protocols

Bone marrow and peripheral blood micronucleus assays were performed simultaneously in the same animals by double dosing regimen (CSGMT, 1990, 1995). Maximum doses established in previous studies (Hayashi et al., 1989 ; CSGMT, 1990, 1992 ; Morita et al., 1997 ; Wakata et al., 1998) were used, and set three lower dose levels at a common ratio of two. Untreated animals as the negative control group and no positive control group were used. Each chemical was administered twice orally, 24 h apart, to 5 rats in each of 3 dosage groups, and bone marrow and peripheral blood were collected 24 h

Table 2 (Continued)

Chemicals	Sex	Dose (mg/kg)	Number of rats	Bone marrow			Peripheral blood			Sex difference	
				MNPCE(%)	MNNCE(%)	PCE(%)	MNRET(%)	MNNCE(%)	RET(%)	MNPCE	MNRET
MMS	♂	10	5	0.21 ± 0.04**	0.00 ± 0.00	73.14 ± 4.21	0.12 ± 0.06**	0.00 ± 0.00	3.52 ± 0.19	M**	M**
		20	5	0.47 ± 0.16**	0.03 ± 0.03	62.90 ± 2.20	0.15 ± 0.04**	0.00 ± 0.00	3.04 ± 0.18*		
		40	5	1.86 ± 0.42**	0.12 ± 0.07**	44.42 ± 5.04**	0.28 ± 0.08**	0.01 ± 0.02	2.08 ± 0.13**		
	♀	10	5	0.18 ± 0.10**	0.00 ± 0.00	56.38 ± 3.24	0.04 ± 0.04	0.00 ± 0.00	2.78 ± 0.27		
		20	5	0.35 ± 0.11**	0.02 ± 0.03	50.34 ± 4.44*	0.05 ± 0.05	0.00 ± 0.00	2.12 ± 0.35		
		40	5	1.67 ± 0.56**	0.10 ± 0.06*	28.12 ± 3.18**	0.23 ± 0.10**	0.00 ± 0.00	1.24 ± 0.28*		
$KBrO_3$	♂	80	5	0.10 ± 0.06	0.01 ± 0.02	58.18 ± 8.48	0.21 ± 0.07**	0.02 ± 0.03	4.34 ± 0.34	ns	ns
		160	5	0.26 ± 0.13**	0.01 ± 0.02	57.48 ± 9.58	0.27 ± 0.12**	0.02 ± 0.03	3.58 ± 0.78		
		320	5	1.75 ± 0.87**	0.11 ± 0.04**	54.00 ± 8.82*	1.41 ± 0.42**	0.07 ± 0.03*	4.76 ± 1.25		
	♀	80	5	0.08 ± 0.03	0.01 ± 0.02	49.00 ± 8.03*	0.17 ± 0.05**	0.01 ± 0.02	3.48 ± 0.51		
		160	5	0.23 ± 0.12**	0.01 ± 0.02	47.48 ± 8.95*	0.24 ± 0.08**	0.03 ± 0.05	2.62 ± 0.86		
		320	5	1.53 ± 0.74**	0.10 ± 0.04*	40.08 ± 8.65**	1.31 ± 0.35**	0.07 ± 0.03*	3.44 ± 1.04		
Urethane	♂	100	5	0.31 ± 0.10**	0.00 ± 0.00	74.78 ± 3.87	0.08 ± 0.03	0.00 ± 0.00	5.26 ± 1.08	ns	M**
		200	5	0.87 ± 0.18**	0.04 ± 0.04	70.44 ± 4.86	0.17 ± 0.06**	0.01 ± 0.02	4.60 ± 1.02		
		400	5	1.49 ± 0.35**	0.06 ± 0.04	51.94 ± 7.77*	0.26 ± 0.11**	0.01 ± 0.02	3.40 ± 0.56		
	♀	100	5	0.37 ± 0.12**	0.01 ± 0.02	56.90 ± 4.07	0.05 ± 0.04	0.00 ± 0.00	3.50 ± 0.53		
		200	5	1.22 ± 0.18**	0.07 ± 0.05*	57.56 ± 2.99	0.14 ± 0.07**	0.00 ± 0.00	3.80 ± 0.75		
		400	5	1.52 ± 0.48**	0.07 ± 0.06*	43.76 ± 4.73**	0.12 ± 0.05**	0.00 ± 0.00	1.80 ± 0.27*		

Data shown in the table are mean ± SD. * $P < 0.05$, ** $P < 0.01$, compared with the results of vehicle control group in each sex.
PCE : polychromatic erythrocytes, RET : reticulocytes, MNPCE : micronucleated PCE, MNRET : micronucleated RET, MNNCE : micronucleated normochromatic erythrocytes. Sex difference : ns ; not significant, M** ; males are significantly more sensitive than females ($P < 0.01$)

later. Acridine orange staining (Hayashi et al., 1983) was used for analysis. The frequency of micronucleated (MN) cells in 2000 polychromatic erythrocytes (PCEs), 2000 normochromatic erythrocytes (NCEs), and 2000 reticulocytes (RETs) were recorded. Cytotoxicity was evaluated by the observation of 300 bone marrow erythrocytes or 2000 peripheral blood reticulocytes.

Statistical analysis

To compare the responses of each dosage group with those of the concurrent control group, Kastenbaum and Bowman's tables (Kastenbaum and Bowman, 1970) were used for the frequency of MNPCEs, MNRETs, and MNNCEs, and a t-test was used for the frequency of PCEs and RETs. To investigate the effect of sex differences, two-way ANOVA test was used for the frequency of MNPCEs and MNRETs.

Results

The results are shown in Table 2.

In the bone marrow assay, the MNPCE frequency increased significantly in all treated animals. There were significant sex differences in the induction of MNPCE in the Ara-C, CP and MMS treated groups, where the magnitude of the increase was higher in males. The MNNCE frequency increased significantly in the CP, MMS, $KBrO_3$, urethane, and Ara-C treated groups but not in the 2-AAF and cholchicine treated groups. The PCE frequency was higher in males in almost all the treatment groups and in the non-treated group.

In the peripheral blood assay, the MNRET frequency increased significantly in the CP, MMS, $KBrO_3$, urethane, and Ara-C treated groups. Sex differences were detected significantly in the MMS and urethane treated groups, where the magnitude of the increase was higher in males than in females. The MNNCE frequency increased significantly only in the $KBrO_3$ treated group. The RET frequency was higher in males in almost all the treatment groups and in the non-treated group.

Discussion

In the bone marrow micronucleus test with MNPCE frequency as the evaluation target, all the chemicals were positive in both sexes but males were significantly more sensitive than females to Ara-C, CP, and MMS. The sex differences might have been caused by differences in chemical bioavailability and metabolism and in erythropoietic function. As for CP, the sex differences may depend on the metabolic activity of cytochrome P450 2C enzyme (CYP2C), which metabolizes CP to a genotoxic intermediate (Clarke and Waxman, 1989). The sex differences in PCE frequency seen in this study may reflect sex differences in erythropoietic function. Nagae et al. (1991) suggest that the induction of micronuclei by mutagens is

inhibited by treatment with estrogen, and this could result in a sex difference in the sensitivity of mice employed in the micronucleus assay. They also suggest mechanisms of the inhibitory effects of estrogen might include a suppression of erythropoiesis. Yajima et al. (1993a, b, c) and Handa and Yajima (1995) speculate that MNPCE frequency increases are the result of errors that occur in the processes of denucleation, differentiation, and repair of genetic damage. They also suggest a close relationship between erythropoiesis and micronucleus induction. The sex differences of body weight may be related to the differences in sensitivity (CSGMT, 1986). When doses were calculated on the body surface, the sex difference tends to lessen (data not shown). When MNNCE frequency was the evaluation target, 5 of 7 chemicals were positive, but only weakly, and evaluation of micronucleus induction using NCEs was inappropriate.

In the peripheral blood micronucleus assay with MNRET frequency as the evaluation target, 2-AAF and cholchicine were negative while the other chemicals were positive in both sexes, with MMS and urethane showing significantly higher responses in the male. Our results reflected that in the rat, the peripheral blood MN assay is less sensitive than the bone marrow MN assay because the rat spleen selectively filters out circulating micronucleated erythrocytes (Schlegel and MacGregor, 1984), especially when the micronuclei are as large as those produced by the spindle poison colchicine. The sex differences in sensitivity may be related to sex differences in erythropoiesis. With MNNCE frequency as the evaluation target, only one chemical ($KBrO_3$) showed positive results, and evaluation of micronucleus induction using NCEs was inappropriate.

In summary, our results showed some sex differences in micronucleus assay sensitivity. These differences may reflect sex differences in erythropoietic function, and the chemical induction of micronuclei in the rat can be evaluated by scoring MNPCEs in the bone marrow or MNRETs in the peripheral blood regardless of sex, with marginal sex differences in sensitivity.

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HIGHER SUSCEPTIBILITY OF NEWBORN THAN YOUNG RATS TO 3-METHYLPHENOL

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ABSTRACT — To determine susceptibility of infants to 3-methylphenol, a repeated dose toxicity study was conducted with oral administration to newborn and young rats. In an 18-day newborn study from postnatal days 4 to 21 at doses of 30, 100 and 300 mg/kg/day, various clinical signs including deep respiration, hypersensitivity on handling and tremors under contact stimulus, and depressed body weight gain were observed at 300 mg/kg. At 100 mg/kg, hypersensitivity and tremors were also noted in a small number of males only on single days during the dosing period. No adverse effects were observed in the 30 mg/kg group. There were no abnormalities of physical development, sexual maturation and reflex ontogeny. The no observed adverse effect level (NOAEL) for newborn rats was considered to be 30 mg/kg/day and the unequivocally toxic level 300 mg/kg/day. In a 28-day study starting at 5 weeks of age, clinical signs and depression of body weight gain, as observed in the newborn rats, appeared in both sexes at 1000 mg/kg but not 300 mg/kg. The NOAEL and the unequivocally toxic level were 300 mg/kg/day and 1,000 mg/kg/day, respectively. From these results, newborn rats were concluded to be 3 to 10 times more susceptible to 3-methylphenol than young rats. However, the realistic no adverse effect dose for the newborn must be slightly lower than 100 mg/kg/day, at which the toxicity incidence was very low, rather than 30 mg/kg/day. Based on this speculation and the equal toxicity at unequivocally toxic levels, the differences in the susceptibility to 3-methylphenol could be concluded to be 3 to 4 times. This is consistent with the results of our previous comparative studies on 4-nitrophenol, 2,4-dinitrophenol and 3-aminophenol, which showed 2 to 4 times differences in the susceptibility between newborn and young rats.

KEY WORDS: Toxicity in newborn rats, 3-Methylphenol

INTRODUCTION

It is known that neonates have specific physiological characteristics with regard to water volume per body, weight of liver and brain relative to body size, cardiac output, respiratory rate, and blood flow to brain and kidney, for example. In fact, the toxicokinetic ability of infants seems to differ from that of adults with respect to their metabolism, clearance, protein binding and volume of distribution, based on data obtained with therapeutic drugs (Besunder *et al.*, 1988; Kearns and Reed, 1989; Morselli, 1989), although there is very little information regarding environmental chemicals. Furthermore, the sensitivity of rapidly developing tissues/systems in neonates may also differ from that in adults (Vesselinovitch *et al.*, 1979; Pope *et al.*, 1991; Faustman *et al.*, 2000). Since infants are always exposed to various chemicals by putting fingers, toys and other objects into their mouths as well as via mother's milk, there is growing concern about effects

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on infant health. Unfortunately, there is no generally accepted Test Guideline for newborn toxicity studies. Therefore, we established a new protocol (Koizumi *et al.*, 2001) in order to investigate the differences of susceptibility between the newborn and young rats. This protocol includes a detailed examination of physical development and sexual maturation, and a complete toxicity analysis after the 9-week recovery-maintenance period, the age of the young adult. Furthermore, a unique feature is that the same lot number of chemical and the same rat strain from the same supplier are used as in young rat studies.

Using this protocol, we have already tested 14 phenolic derivatives as a part of an existing chemical testing program of Japan in 1999. So far, we have reported three comparative analyses of 4-nitrophenol, 2,4-dinitrophenol and 3-aminophenol (Koizumi *et al.*, 2001, 2002a, 2002b; Yamamoto *et al.*, 2001; Takano *et al.*, 2001; Nishimura *et al.*, 2002). In these studies, for more precise / appropriate comparison, we estimated both the no observed effect levels (NOAELs) and unequivocally toxic levels, defined as doses inducing severe toxic signs including death or critical histological damage, based on the results of both the main studies and the dose-finding studies for each case. In consequence, it was concluded that the susceptibility of newborn rats to the toxicity of these chemicals ranged from 2 to 4 times that of young rats.

In the present study, we selected 3-methylphenol, widely known as m-cresol and used in synthetic resins, disinfectants and pharmaceutical raw materials (Chemical Products' Handbook, 2002). Several reviews on the toxicity of this chemical or cresols, including three isomers, have been published (ATSDR, 1991; EHC, 1995; IRIS, 1997). For 3-methylphenol, although various clinical signs, growth inhibition and some developmental effects have been reported (TRL, 1986; BRRC, 1988a, 1988b, 1989; MBA, 1988; NTP, 1992), there are no data to our knowledge on its direct effects in newborn animals. In this study, we estimated the NOAELs and unequivocally toxic levels of 3-methylphenol, and compared them between newborn and young rats employing the previously described protocol.

MATERIALS AND METHODS

Materials

3-Methylphenol (CAS No. 108-39-4, purity: 99.13%) was obtained from Honshu Chemical Industry Co., Ltd. (Wakayama, Japan), and dissolved in olive oil. The test solution was prepared at least once a week and kept cool and in the dark until dosing. The stability was confirmed to be at least 8 days under these conditions. All other reagents used in this study were specific purity grade.

Animals

Sprague-Dawley SPF rats [Crj:CD(SD)IGS] were purchased from Charles River Japan Inc. (Kanagawa, Japan) and maintained in an environmentally controlled room at 20-26°C with a relative humidity of 45-65%, a ventilation rate of more than 10 times per hour, and a 12:12 hr light/dark cycle. In the 18-day main study of newborn rats, 21 pregnant rats (gestation day 15) were purchased and allowed to deliver spontaneously. Among all newborns separated from dams at postnatal day 3 (the date of birth was defined as postnatal day 0), 48 males and 48 females were selected by stratified random sampling based on the body weight and assigned to 4 dose groups, including controls. Twelve foster mothers suckled the 4 males and 4 females assigned to each group up to weaning on postnatal day 21 (termination of dosing). After weaning, the animals of the recovery-maintenance group were individually maintained for 9 weeks. In the 28-day study of young rats, 4-week-old male and female rats were obtained and used at ages of 5 weeks after acclimation. All animals were allowed free access to basal diet (newborn rat study: LABO MR stock, Nihon Nosan Kogyo Inc., Yokohama, Japan; young rat study: CRF-1, Oriental Yeast Co. Ltd., Tokyo, Japan) and tap water.

Study design (Time schedule as reported previously (Koizumi *et al.*, 2001))

1. 18-Day repeated dose study in newborn rats

1) Dose-finding study

Newborn rats (5/sex/dose) were administered the test substance at 0, 100, 300 or 1,000 mg/kg/day in olive oil by gastric intubation daily from postnatal days 4 to 21. They were examined for general behavior and body weights during the dosing period, and sacrificed at postnatal day 22 after overnight starvation, for assessment of hematology, blood biochemistry, macroscopic findings and organ weights.

2) Main study

Newborn rats (12/sex/dose) were administered 3-methylphenol at 0, 30, 100 or 300 mg/kg/day in olive oil by gastric intubation daily from postnatal days 4 to 21, based on results of the dose-finding study, and 6

males and 6 females in each group were sacrificed on postnatal day 22 after overnight starvation. Recovery-maintenance groups (rest of animals in all groups) were maintained for 9 weeks without chemical treatment and fully examined at 12 weeks of age. General behavior was noted at least once a day for newborn rats (separated from each foster mother) and foster mothers. Body weight was measured at postnatal days 4, 7, 10, 13, 16, 19 and 21, and then at 7-day intervals, and food consumption (for 24 hr from the day before) at the same days after weaning. At postnatal day 20 for males and day 21 for females, gait condition, pupillary reflex, auricular reflex, corneal reflex, visual placing reflex, surface and mid-air righting reflexes, and ipsilateral flexor reflex were examined. Furthermore, fur appearance, incisor eruption and eye opening were observed in all animals from postnatal days 7, 9 and 11, respectively, and testes descent and vaginal opening were examined from postnatal days 17 and 29, respectively. Color, pH, occult blood, protein, glucose, ketone bodies, bilirubin, urobilinogen, sediment, specific gravity and volume of the urine were examined only at the end of the recovery-maintenance period. Blood was collected from the abdominal aorta under ether anesthesia at sacrifice after overnight starvation for scheduled-sacrifice and recovery-maintenance groups. One part was treated with EDTA-2K or 3.8% sodium citrate and examined for hematological parameters such as the red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count, platelet count, reticulocyte count, and leukocyte analysis percentage, as well as blood clotting parameters such as prothrombin time and activated thromboplastin time. Serum obtained from another portion of the blood was analyzed for blood biochemistry (total protein, albumin, albumin-globulin ratio, glucose, total cholesterol, triglycerides, phospholipid, total bilirubin, urea nitrogen (BUN), creatinine, glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), alkaline phosphatase, γ-glutamyl transpeptidase (γ-GTP), lactate dehydrogenase, cholinesterase, calcium, inorganic phosphorus, sodium, potassium, chlorine). After gross examination of the body surface, orificial mucosa and internal organs of animals sacrificed by exsanguination following collection of blood, the brain, pituitary gland, thymus, thyroids, heart, lungs, liver, spleen, kidneys, adrenals, testes, epididymides, prostates, seminal vesicle, ovaries and uterus were removed and weighed. Histopathological examination was conducted for the control and the highest dose groups. The trachea, stomach, intestine, pancreas, lymph glands, urinary bladder, spinal cord, bone marrow and sciatic nerve as well as the above organs were fixed in 10% buffered formalin-phosphate (following Bouin's fixation for testes and epididymides), and paraffin sections were routinely prepared and stained with Hematoxylin-Eosin for microscopic examination. For other groups, the organs with macroscopically abnormal findings or in which dose-related effects were evident on microscopic examination for the highest dose group, were examined.

2. 28-Day repeated dose study in young rats

1) Dose-finding study (14-day study)

Five-week-old rats (5/sex/dose) were administered the test substance at 0, 125, 250, 500 or 1,000 mg/kg/day in olive oil by gastric intubation for 14 days. They were examined for general behavior, body weight and food consumption during dosing and sacrificed after overnight starvation following the last treatment for assessment of hematology, blood biochemistry, macroscopic findings and organ weights.

2) Main study

Five-week-old rats were given the test substance in olive oil by gastric intubation daily for 28 days and sacrificed after overnight starvation following the last treatment. Referring to the results of the dose-finding study, 4 doses, including the control, were established (0, 100, 300, 1,000 mg/kg/day). Recovery groups (0, 1,000 mg/kg) were maintained for 2 weeks without chemical treatment and fully examined at 11 weeks of age. The number of animals for each sex/dose was 7 for both scheduled-sacrifice and recovery cases. Rats were examined for general behavior, body weight, food consumption, urinalysis, hematology and blood biochemistry, necropsy findings, organ weights and histopathological findings in compliance with the Test Guideline of the Japanese Chemical Control Act (Official Name: Law Concerning the Examination and Regulation of Manufacture, etc. of Chemical Substances) under Good Laboratory Practice conditions.

Statistical analysis

Data were statistically analyzed as follows (Sakuma, 1977, 1981; Yamasaki *et al.*, 1981). Continuous data were analyzed by Bartlett's test for distribution. When homogeneity was recognized, one-way analysis of variance was performed. When a significant difference was observed, Dunnett's or Scheffe's tests