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Three Rs in mutation research
-- From *in vivo* to *in silico* evaluation --

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Abstract

Many genotoxicity assay systems have been developed and introduced for safety assessment of chemicals. More than a half of them are *in vitro* systems therefore we can say that the field of genotoxicity started from the alternatives of animal experiments. Our respect to the 3Rs, we are introducing the *in silico* evaluation system, reconsidering the interpretation of positive outcomes from the *in vitro* assay systems based on the weight of evidence, and incorporating *in vivo* genotoxicity assay systems into general toxicological studies. These attempts replace existing systems with new systems without using any animals, reduce number of animals because of reduction of irrelevant *in vitro* positives, and share animals with other study as multiple endpoint assay systems.

Keywords: genotoxicity, micronucleus assay, *in silico*

1. Introduction

In this article, "mutation" is defined as a permanent change in the amount or structure of the genetic material of an organism, which may result in a heritable change in the characteristics of the organism. "Genotoxicity" is a broad term that includes mutation as well as interaction to DNA and/or the cellular apparatus which regulates the fidelity of the genome, *e.g.*, the spindle apparatus, and enzymes such as the topoisomerases, by the chemical itself or its metabolites. It includes UDS, SCE, and mitotic recombination. In other words, I will use the term "mutagenicity" restricting to only gene mutation and structural and numerical chromosomal aberrations, while Genotoxicity consists of mutagenicity, DNA adduct formation, DNA damage (UDS, Comet), sister chromatid exchange (SCE), etc.

Many assay systems have been developed and introduced for safety assessment of chemicals. Table 1 shows the assay systems grouped by the endpoints and materials. More than a half of them are *in vitro* assay systems therefore we can say that the field of genotoxicity started from the alternatives of animal experiments. Although there are many kinds of assay systems but none can detect chemical genotoxicity. Assays are generally endpoint specific, so we usually use several assays in combination referred to as "battery".

The standard test battery for genotoxicity approved by ICH for pharmaceutical drugs in 1997 is as follows (Müller et al., 1999):

i) A test for gene mutation in bacteria
ii) An *in vitro* test with cytogenetic evaluation of chromosomal damage with mammalian cells or an *in vitro* mouse lymphoma tk assay
iii) An *in vivo* test for chromosomal damage using rodent haematopoietic cells

Two most important endpoints are "gene mutation" and "chromosomal aberration". This battery was proposed for pharmaceutical drugs in international harmonization. This also includes gene mutation and chromosomal aberration *in vitro*, and one *in vivo* assay.

2. The limitation of *in vitro* genotoxicity assay

We have a long history to make safety assessment using *in vitro* assays. Based on our experience, we understand the limitation of the *in vitro* assays. For example, Fig. 1 shows the percentages of registered industrial chemicals that showed positive, equivocal, and negative. By the Ames test, only 11% of chemicals showed positive, while more than 30% showed positive by the chromosomal aberration test. Including equivocal, almost 40% chemicals showed some responses in induction of structural and/or numerical chromosomal aberration. Some of the positive responses are known that they are not biologically relevant. Kirkland et al. (2005) published a paper providing extensive database on *in vitro* assay outcomes for carcinogens and also for non carcinogens. This paper had an impact to trigger the discussion of interpretation of *in vitro* genotoxicity assay data especially positive ones. There are several movements on the international level. One of them was the ECVAM Workshop on "How to reduce false positive results with *in vitro* genotoxicity testing and

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Table 1. Genotoxicity assays

	In vitro	*In vivo*
DNA interaction	Covalent binding ^{32}P post labeling Oxidative damage (*e.g.*, 8-OH-dG) SCGE (Comet)	Covalent binding ^{32}P post labeling Oxidative damage (*e.g.*, 8-OH-dG) SCGE (Comet)
DNA repair	*rec*-assay, *pol*-assay, *umu*-assay, SOS-assay unscheduled DNA synthesis (UDS) assay	unscheduled DNA synthesis (UDS) assay
Gene mutation	Bacterial reverse mutation assay (e.g., Ames test) *D. melanogaster* sex-linked recessive lethal assay Mammalian cell gene mutation (e.g., MLA)	Mouse specific locus, coat color spot Transgenic animal model
Chromosomal damage	Aneuploid assay in yeast *In vitro* metaphase analysis (mammalian, human cells) *In vitro* micronucleus test	*In vivo* micronucleus test *In vivo* metaphase analysis Dominant lethal test Specific locus assay Reciprocal transformation
Others	Gene conversion in yeast Sister chromatid exchange assay Morphological cell transformation	Sister chromatid exchange assay

Table 2. Model chemicals used in the validation study

Alkylating agents Cyclophosphamide monohydrate Dimethylnitrosamine Ethyl methanesulfonate *N*-Ethyl-*N*-nitrosourea Methyl methanesulfonate *N*-Methyl-*N'*-nitro-*N*-nitroso-guanidine Triethylenemelamine **Base analogues and related chemical** 1-β-D-arabinofuranosylcytosine 5-Fluorouracil 6-Mercaptopurine Methotrexate **Aromatic amines** 2-Acetylaminofluorene Phenacetin	**Polycyclic aromatic hydrocarbons** Benzo[*a*]pyrene 7,12-Dimethylbenz[*a*]anthracene **Crosslinking agent** Mitomycin C **Inorganic chemicals** Potassium bromate Potassium chromate (VI) **Spindle poisons** Colchicine Vincristine sulfate **Miscellaneous chemicals** Benzene Procarbazine hydrochloride Urethane

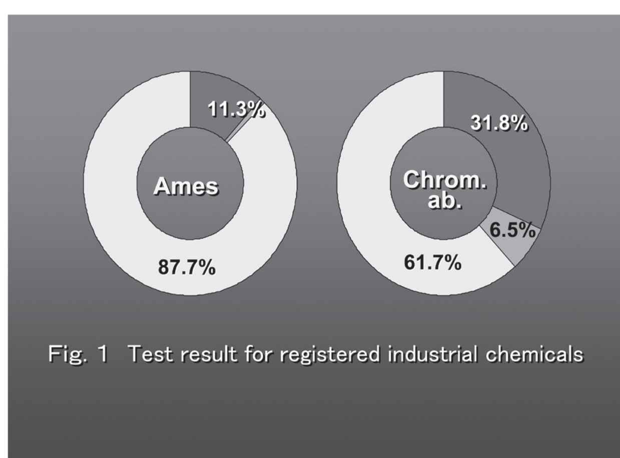

Fig. 1 Test result for registered industrial chemicals

avoid unnecessary follow-up animal tests". It was held in Ispra, Italy on April 27 and 28, 2006 (Kirkland et al., 2007). Another one is the IWGT, International Workshop on Genotoxicity Testing. We started this international workshop in 1983 and focused mainly technical aspects of the tests but we started to discuss on the strategic issue including the topic of irrelevant *in vitro* positive outcomes in the 3rd (Müller et al., 2003) and 4th (Thybaud et al., 2007).

Another one is the activity by International Life Science Institute/Health and Environmental Sciences Institute (ILSI/HESI). They started the emerging issues subcommittee focused on "the relevance and follow-up of positive results in *in vitro* genetic toxicity (IVGT) testing" (Thybaud et al., 2007). These activities all agreed that there are significant numbers of irrelevant positive outcomes from the *in vitro* mammalian cell assays. If we can avoid such irrelevant positives then we can reduce the follow up *in vivo* assays reading the reduction of unnecessary animals.

3. Reduction of animals—in the case of the rodent micronucleus test

We have tried to reduce animals without loosing scientific value of the test. One of the most important endpoints of genotoxicity is an induction of chromosomal aberration. As mentioned above, there are many positives in *in vitro* chromosomal

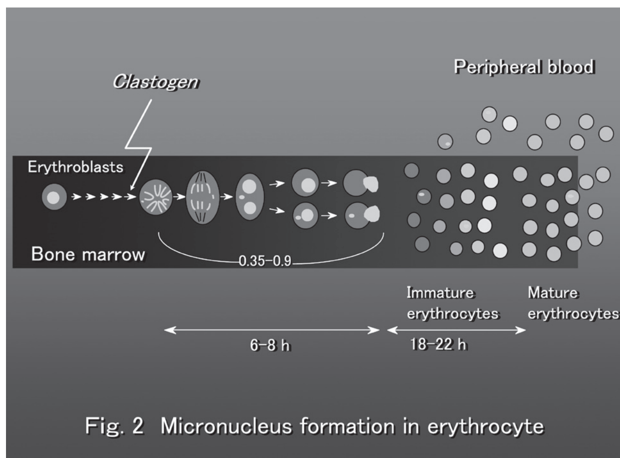

Fig. 2 Micronucleus formation in erythrocyte

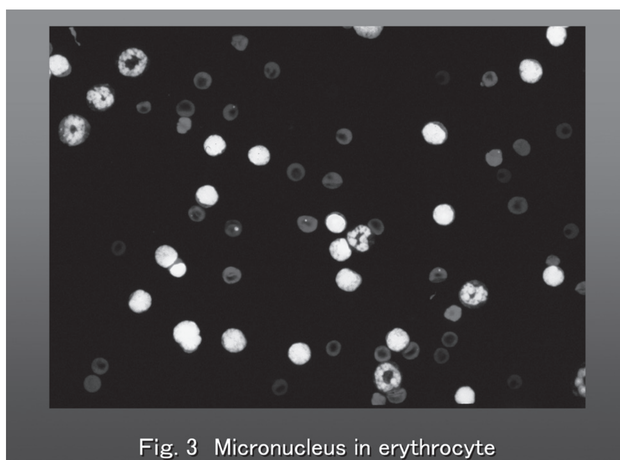
Fig. 3 Micronucleus in erythrocyte

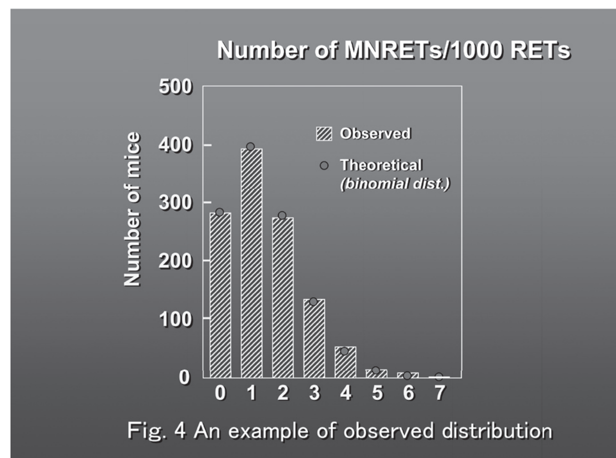

Fig. 4 An example of observed distribution

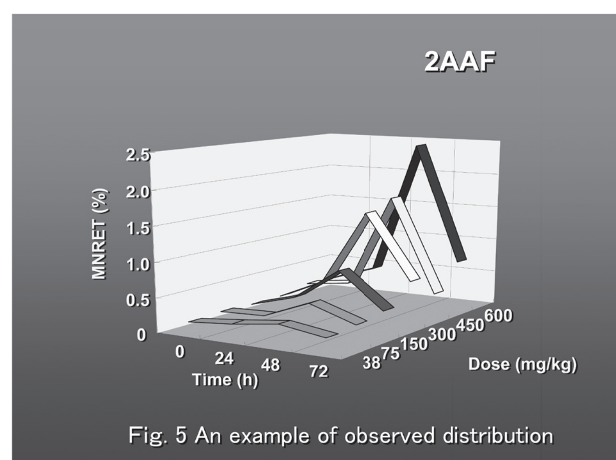

Fig. 5 An example of observed distribution

aberration assays. Accordingly, to assess the relevancy of such effects should be evaluated by *in vivo* cytogenetic assay, such as the rodent bone marrow micronucleus assay. To assess the ability to induce chromosomal aberration in *in vivo*, the rodent micronucleus test has been used most widely and frequently. Fig. 2 shows a schematic model to explain how micronuclei are formed in correlation to the chromosomal aberration (Hayashi et al., 1984). When we see the small nucleus in red blood cells, it is a good indication of chromosomal aberration induced during erythropoiesis because there is no nucleus in the mammalian erythrocyte. Fig. 3 is a representative microphotograph of mouse bone marrow cells. Historically, we used bone marrow cells for this test and we had to kill animals. We thought that we had to evaluate the existence of micronucleus in the young red blood cells in the bone marrow before dilute out with many existing cells in the peripheral blood stream. We, however, found that young red blood cells in the peripheral blood stream can also be targeted cells for analysis in the micronucleus test as well as in the bone marrow. I applied a supra-vital staining method using acridine orange to this test (AO coat-slide method, Hayashi et al., 1990). An extended validation study on the AO coat-slide method was organized by Collaborative Study Group for the Micronucleus Test, which belong to the Mammalian Mutagenicity Study group, which is a sub-organization of Japanese Environmental Mutagen Society (CSGMT/MMS/JEMS) (CSGMT, 1992). Forty four laboratories, industries, CROs, universities, and public research laboratories participated to the validation study. It also included laboratories from overseas countries. We selected 23 model chemicals with variety modes of action to induce chromosomal aberrations (Table 2).

Fig. 4 is a distribution of observed number of mice with micronucleated young red blood cells in the control group obtained in this collaborative validation study; observed frequencies are well fitted to the theoretically calculated binomial distribution. This means the method has little factors that can be variable. Fig. 5 is an example of outcomes on a model chemical (2-acetylaminofluorene, 2-AAF). Taking a very tiny volume of peripheral blood, we need 5 (or less) micro-liters of blood, and we do not have to kill animals. Thus, we can get information from the same animal at different timings from the treatment. As a rule, we study one chemical by at least two laboratories and evaluate reproducibility between laboratories. All model clastogens and aneugens induced micronuclei as expected and observed in the peripheral blood (CSGMT 1992). OECD and other test guidelines suggested the size of the micronucleus test for bone marrow assay as shown in the left side of Table 3, while that for peripheral blood MN assay as in the right side. We need 25 or 45 animals

Table 3. Number of animals recommended in MN assays

Size of bone marrow MN assay	Size of peripheral blood MN assay
■ 5 animals/group ■ -ve, +ve control and ≥ 3 treatment groups ■ Single treatment and 2 sampling times (or twice or more treatments and single sampling time) ■ If equivocal then re-test At least 25 animals (double treatment) At least 45 animals (single treatment)	■ 5 animals/group ■ ≥ 3 treatment groups ■ Single treatment and 2 sampling times (or twice or more treatments and single sampling time) ■ If equivocal then re-test At least 15 animals (double treatment) At least 15 animals (single treatment)

Table 4. Evaluation data set and *in silico* models

Evaluation data set:

Database of existing chemicals in Japan evaluated by GLP test

	Ames test	*Chromosomal aberration*
Positive:	26	98
Negative:	180	121
Total:	206	219

***In silico* systems used:**

DEREK; MultiCase (MCase+); AdmeWorks

Table 5. Combination of *in silico* outcomes

		In silico		Total	
		++	~		
Ames	+	19	7	26	Sensitivity 73.1%
	–	23	147	170	Specificity 86.5%
	Total	42	154	196	Concordance 84.7%
	Applicability 95.1% (196/206)				

are required for the ordinal method in the double or single treatment protocol, respectively. However, when we use the peripheral blood MN assay, we need only 20 animals regardless the number of treatment, because we do not need to set a negative control group because pretreatment animals can serve as a negative control. In 1999 Washington DC meeting, we discussed and proposed the possibility for the MN test without positive control. Although there are several conditions to omit the concurrent positive control group, there is big possibility to operate the MN test without concurrent positive control group. Then, we can perform the MN test using only 15 (1/3 of BM, single treatment) animals are needed to evaluate chromosomal aberration induction without loosing any significant information.

The method can also be incorporated into general toxicological assay (Hamada et al., 2001). This means that the experimental animals can be shared for assays to examine different endpoints and can be reduced animals in total for the assessment of chemical safety.

4. *In silico* evaluation of mutagenesis

The ultimate 3Rs of the animal experiments is the *in silico* system for the genotoxicity evaluation. There are thousands of chemicals around us in our daily life. Only, however, small part of them have been assessed their safety. To evaluate the resting chemicals of their toxicity, if we will evaluate by testing, even short-term tests, it will take more than hundred years. We need more efficient and high-throughput methods for evaluation of chemical safety. We have studied the chemical genotoxicity, gene mutation and chromosomal aberration, by (Q)SAR models. We studied on this project supported by MHLW grant. We used 3 commercially available (Q)SAR models, namely DEREK, MultiCase, and AdmoWorks, which is a Japanese model. As evaluation datasets, we used 206 chemicals for Ames assay and 219 for chromosomal aberration assay, which were existing chemicals in Japan and recently evaluated mutagenicity by GLP testing (Table 4).

We trained these (Q)SAR models individually using learning dataset, but the performance showed some limitation for each models when used individually. On the other hand, when we evaluate in combination of these models to evaluate mutagenesis we could obtain reliable outcomes. Table 7 shows the outcomes if we judge bacterial mutagenesis positive by *in silico* only when two or more models evaluated positive. And for negative, two or more models revealed negative then final judge was negative. Table 5 shows the correlation between results of Ames test and *in silico* evaluation: the sensitivity was 73%, specificity was 86.5A%, and total concordance was 84.7%. The applicability was 95%, which means 95% of chemicals could be evaluated.

Table 6 shows the result under more strict condition. Chemicals are judged positive only when all three models showed positive evaluation, and negative only when all showed negative evaluation. All sensitivity, specificity, and concordance gave very high values, but the applicability became lower to only 55.3%. In summary, a good concordance could be obtained using this strategy, but the applicability was reduced to near 1/2 (Hayashi et al., 2005).

We are applying this strategy to the *in vitro* chromosomal aberration and obtaining the same

Table 6. Combination of *in silico* outcomes

		In silico		Total	
		+++	−−−		
Ames	+	13	2	15	Sensitivity 86.7%
	−	5	94	99	Specificity 94.9%
	Total	18	96	114	Concordance 93.9%
	Applicability 55.3% (114/206)				

Table 7. Examples of genotoxicity tests

	DNA damage	Gene mutation	Chrom. aberration
In vivo	Rat Comet	TG rat model	Rat PB MN
In vitro	Tk6 cell Comet	Tk6 cell mutation	Tk6 cell MN
In silico	DNA bind. A. ToolBox	Combined model	Combined model

tendency to the Ames test.

5. Concluding remarks

We have many assay systems to assess the genotoxicity of chemicals. Table 7 shows examples for *in vivo, in vitro*, and *in silico* assays. In the field of genotoxicity, we started using *in vitro* systems. After quite long period, our experience showed the limitation of *in vitro* systems, and now we are developing the *in silico* applications. Our respect to the 3Rs, we started to introduce the *in silico* evaluation system, to reconsider the interpretation of positive outcomes from the *in vitro* assay systems based on the weight of evidence, and to incorporate *in vivo* genotoxicity assay systems into general toxicological studies. These attempts replace existing systems with new systems without using any animals, reduce number of animals because of reduction of irrelevant *in vitro* positives and also share animals because of multiple endpoint assay systems. We have to use any kind of information effectively using full of our wisdom. Finally, I would like to cite my favorite words by Dr. Torahiko Terada and translated by Dr. Sohei Kondo "*It is very easy to over- or under-estimate of risk but very hard to make a rational assessment of risk*".

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