

Assessing the potential mutagenicity of pesticides

by James M Parry, DSc¹

Parry JM. Assessing the potential mutagenicity of pesticides. *Scand J Work Environ Health* 2005;31 suppl 1:123–128.

Determining mutagenic profiles of pesticides requires tests of high sensitivity and specificity. An effective strategy uses tests that produce reproducible and biologically relevant data based upon three stages. Stage 1, in vitro, uses (i) bacterial gene mutation assays, (ii) assays measuring clastogenicity and aneugenicity, and (iii) assays measuring the induction of gene mutations in cultured mammalian cells. Stage 1 can detect most mutagenic hazards. Stage 2, in vivo testing in somatic cells of rodents, is required to determine whether in vitro positives are reproduced in vivo and to detect activity only produced in intact animals. Decisions on assay selection should be based on the in vitro profile. In most cases in vivo assessment is based on the micronucleus assay in rodent bone marrow. Stage 3, in vivo germ-cell testing, is rarely required for pesticides that have been shown to be mutagenic in somatic cells in vivo.

Key terms aneugen; clastogen; genotoxicity; review; strategy.

The growth of interest in the potential mutagenicity of chemicals in the 1970s and 1980s resulted in the development of more than 90 test systems. Many of the developed test systems are better described as “genotoxicity test systems” because they evaluate the induction of end points broader than the production of inherited changes in the genetic material of a cell. The developed methods range from the use of viruses to those involving intact rodents, and they cover both the measurement of mutagenic activity itself and a range of indirect end points including DNA (deoxyribonucleic acid) adduct formation, DNA repair activity, and the production of chromosomally aberrant rodent embryos. Table 1 illustrates the variety of test methods that have been approved by the European Union for assessing the mutagenicity of chemicals, including pesticides. Many of these assays currently have only a limited use or may only be available in a few laboratories.

A consequence of the proliferation of test systems was their application in investigating the potential mutagenicity of various types of pesticides, including both active ingredients and various commercial preparations. For example, the fungicide carbendazim has been evaluated for mutagenic potential in more than 30 assay systems and the herbicide chlorpropham has been assessed in more than 50 assay systems. A result of these studies

has been the development of data sets that show confusing patterns of positive and negative results. When the potential mutagenic activity of a pesticide is evaluated, there are two prime considerations, which assay systems should be selected for use and how the selected test

Table 1. The genotoxicity tests listed in annex V of European Union directive 6/548. (DNA = deoxyribonucleic acid)

Code	Test
B.10	Mutagenicity—in vitro mammalian chromosome aberration test.
B.11	Mutagenicity—in vitro mammalian bone-marrow chromosome aberration test.
B.12	Mutagenicity—mammalian erythrocyte micronucleus test
B.13/14	Mutagenicity—reverse mutation test using bacteria
B.15	Gene mutation— <i>Saccharomyces cerevisiae</i>
B.16	Mitotic recombination— <i>Saccharomyces cerevisiae</i>
B.17	Mutagenicity—in vitro mammalian cell gene mutation test
B.18	DNA damage and repair—unscheduled DNA synthesis—mammalian cells in vitro
B.19	Sister chromatid exchange assay in vitro
B.20	Sex-linked recessive lethal test in <i>Drosophila melanogaster</i>
B.21	In vitro mammalian cell transformation test
B.22	Rodent dominant lethal test
B.23	Mammalian spermatogonial chromosome aberration test
B.24	Mouse spot test
B.25	Mouse heritable translocation
B.39	Unscheduled DNA synthesis (UDS) test with mammalian liver cells in vivo

¹ Centre for Molecular Genetics and Toxicology, School of Biological Sciences, University of Wales Swansea, Singleton Park, Swansea, United Kingdom.

Reprint requests to: JM Parry, Centre for Molecular Genetics and Toxicology, School of Biological Sciences, University of Wales Swansea, Singleton Park, Swansea, SA2 8PP, UK. [E-mail: JMP@swansea.ac.uk]

systems can be used to develop an effective testing strategy capable of both detecting most, if not all, potential mutagens and indicating whether any activity detected is biologically relevant to nontarget (in the case of pesticides) species, including humans.

The aims of mutagenicity testing programs can be varied, and I have focused in this report on the development and application of a testing strategy that is (i) capable of detecting intrinsic mutagenic potential, an exercise that can primarily be based on the use of *in vitro* test systems, (ii) capable of determining whether mutagenic potential detected *in vitro* is also reproduced *in vivo*, and (iii) capable of determining whether, if mutagenic hazard is detected *in vivo*, the activity represents a potential hazard to somatic cells such as the development of cancer or to germ cells such as an elevation in inherited defects.

The development of such an effective testing program requires a strategy that primarily utilizes assay systems that have the following key properties: (i) good validation, preferably at the international level, (ii) methods that are widely used and have a large database for comparative purposes, (iii) methods that are well characterized in terms of their sensitivity and reliability, (iv) methods that have a high sensitivity and thus detect most, if not all, potential mutagenic hazards, and (v) methods that have a high level of specificity and thus avoid the generation of a high level of false positive results.

The balance between specificity and sensitivity can be difficult to achieve, but, in general, for suitable methods, guidelines of the Organization for Economic Cooperation and Development (OECD) are available that describe the necessary criteria for optimal test performance.

Strategy for the mutagenicity testing of pesticides

In 2000 the Advisory Committee on the Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM) in the United Kingdom initiated an exercise to develop a strategy of mutagenicity testing that involved a review of the application of the currently available test systems that could be considered suitable for the assessment of a wide range of chemicals (including pesticides). The conclusions of this exercise were published in December 2000 (1).

As indicated earlier, more than 90 assay methods can potentially be used to identify the genotoxic hazard of chemicals. However, the COM decided that its recommended strategy of mutagenicity testing should be based on the use of a limited number of well-validated and informative test systems.

A key feature in the selection and application of mutagenicity test systems is that they should be capable of providing information on the three basic levels of mutation that are relevant to the induction of both cancer and birth defects, namely, gene or point mutation, clastogenicity (the induction of structural chromosome aberrations), and aneugenicity (the induction of chromosome numerical aberrations).

This selection of mutagenicity end points is more focused and biologically relevant than those covered by the term genotoxicity, which has generally been used to cover any effect that has the potential to modify the integrity of the genome.

The mutagenicity testing strategy recommended by COM is based on the following three basic stages: (i) stage 1—*in vitro* evaluation of the potential of a pesticide to induce point or gene mutations and the induction of chromosome aberrations of structure and number, (ii) stage 2—*in vivo* testing in somatic cells with two aims (ie, to determine whether *in vitro* activity is reproduced *in vivo* and to determine whether a pesticide has *in vivo* mutagenic activity that was not detected under *in vitro* test conditions), and (iii) stage 3—*in vivo* testing in germ cells to determine whether *in vivo* somatic cell activity is reproduced in germ cells and thus represents the potential to induce inherited defects.

This separation of the stages of mutagenicity testing is important as European Union directive 93/21/EEC classifies mutagenic hazard into the following three basic categories: category 1:90, substances known to be mutagenic to man (there is sufficient evidence to establish a causal association between human exposure to a substance and heritable genetic damage); category 2:91, substances that should be regarded as if they are mutagenic to man (there is sufficient evidence to provide a strong presumption that human exposure to the substance may result in the development of heritable genetic damage, generally on the basis of appropriate animal studies and other relevant information); category 3:93, substances that cause concern for humans owing to possible mutagenic effects (there is evidence of activity from appropriate mutagenicity studies but this is insufficient to place the substance in category 2).

The mutagenicity testing strategy developed by COM will provide information relating to the European Union classification categories in a stepwise manner by following the following three stages of testing: (i) stage 1, the *in vitro* methodologies recommended by COM; (ii) stage 2, the strategy for the *in vivo* testing of pesticides in germ cells; and stage 3, the strategy for the *in vivo* testing of pesticides in germ cells.

Stage 1, the *in vitro* methodologies recommended by COM, involves three types of methods. The first is a bacterial test for gene mutation, the use of bacterial assays for gene mutations (point, insertions, deletions)

using specialized strains of *Salmonella typhimurium* or *Escherichia coli*. The tests should be performed in the presence and absence of an exogenous source of mammalian metabolic activity, generally liver S9 mix from a rat treated with an enzyme inducing preparation. The second includes tests for clastogenicity and aneugenicity, the current methodology recommended by the Organisation for Economic Co-operation and Development (OECD) involving the analysis of metaphase cells generated from cell cultures derived from permanent cell lines (generally from Chinese hamster cultures) or from human lymphocytes from an "appropriate" donor (2). These assays provide information on changes in the structure and number of chromosomes. The third is the mammalian cell mutation assay. Although a range of point or gene mutations assays is available, COM expressed its preference for the mouse lymphoma L5178Y assay, which measures the induction of drug resistance due to the homozygosity of defective alleles of the thymidine kinase gene. This assay has been extensively validated and has adequate "statistical power" to allow the detection of weak mutagenic activity.

In the case of the selection of an in vitro assay for the detection of the potential clastogenicity and aneugenicity of chemicals, COM exposed a preference for the use of the in vitro binucleate cell micronucleus assay originally developed by Fenech and his colleagues (3, 4). In this assay, cultures treated with a test pesticide undergo nuclear division in the presence of the actin inhibitor cytochalasin B, which prevents the completion of cytokinesis and thus produces binucleate cells. Micronuclei are produced by chromosome breakage (clastogenicity) or the failure of individual chromosomes to segregate into either of the two nuclei of the binucleate cell (chromosome loss as an indicator of induced aneuploidy). Micronuclei containing chromosome acentric fragments (produced by breakage) can be distinguished from those containing whole chromosomes (produced by chromosome loss) by the use of an antibody to kinetochore proteins, which are present at the centromere of the segregating chromosome, or by the use of molecular probes for the centromere region itself. The in vitro binucleate cell assay can also be used to measure the induction of the malsegregation of chromosomes leading to nondisjunction, which produces nuclei that contain either a missing or extra chromosome by the use of centromere probes for specific chromosomes. The combination of the various developments of the in vitro binucleate cell assay provides a powerful tool for identifying clastogenic and aneugenic (both chromosome loss and nondisjunction) activity in vitro.

An example of the application of the in vitro binucleate cell assay in the assessment of the mutagenic potential of the organophosphate insecticide trichlorfon is illustrated next. Although trichlorfon has been implicated

in the etiology of a cluster of congenital birth abnormalities (including Down's syndrome) in Hungary by Cziezel and his colleagues (5), there is no comprehensive database for trichlorfon over the three stages of mutagenicity testing recommended by COM.

Table 2 illustrates the effects of trichlorfon on the cultured human lymphoblastoid cell line AHH-1, as measured in the in vitro binucleate cell assay. The data presented demonstrate that trichlorfon induced dose-dependent reductions in the frequency of binucleate cells (a measure of toxicity) and increases in micronuclei frequencies in binucleate cells (a measure of clastogenic and aneugenic activity) when cells were exposed for 6 hours at pH 5.5.

Samples of micronucleated cells were treated with an antibody specific for the centromeres of all human chromosomes, and 1000 micronuclei per treatment were screened for centromere-positive micronuclei containing whole chromosomes and centromere-negative signals for acentric chromosome fragments. In control cultures, the relative proportion of centromere-positive micronuclei containing whole chromosomes was 56%. After exposure to trichlorfon at concentrations of up to 20 µg/ml the percentage of centromere-positive micronuclei increased to between 73% and 78%, indicating the induction of chromosome loss suggesting that trichlorfon showed aneugenic activity. In contrast, at a toxic dose of 80 µg/ml, the percentage of centromere-positive micronuclei fell to 39%, indicating that 61% of the micronuclei contains acentric chromosome fragments. Such observations indicate that trichlorfon has clastogenic activity at toxic doses.

To confirm the aneugenic potential of trichlorfon seen at low exposure doses, cells were treated with centromere-specific molecular probes for human chromosomes 2, 7, and 18. Altogether 1000 cells per treatment were screened for the distribution of chromosomes into the daughter nuclei of binucleate cells. When cells undergo cell division with normal chromosome segregation (disjunction), the daughter nuclei each receive two copies of each chromosome (2:2 segregation).

Table 2. Demonstration of the induction of micronuclei in a binucleate cell assay using the cultured human lymphoblastoid cell line AHH-1 treated with the insecticide trichlorfon for 6 hours at pH 5.5. ^a

Concentration of trichlorfon	Binucleate cells (%)	Micronucleated binucleate cells (%)	Centromere positive micronuclei (%)
0 µg/ml	41.5	1.85	56
5 µg/ml	31.5	3.55 ^b	78
10 µg/ml	27.2	5.50 ^b	75
20 µg/ml	22.9	6.60 ^b	73
40 µg/ml	15.6	7.40 ^b	49
80 µg/ml	10.3	10.20 ^b	39

^a Data kindly supplied by Dr Anne Doherty.

^b P<0.05.

Table 3 illustrates the frequencies of cells that contain abnormal numbers of chromosomes 2, 7, and 18 (ie, they have undergone nondisjunction). The data illustrate an increase in nondisjunction, which occurred in a dose-dependent manner up to 20 µg/ml at all treatment doses of trichlorfon. The frequencies of nondisjunction fell at the higher doses, for which there was substantial toxicity. These data confirm the aneugenic activity of trichlorfon with both chromosome loss and nondisjunction activity detected at exposure doses of up to 20 µg/ml.

The *in vitro* studies thus demonstrate that trichlorfon has aneugenic activity at nontoxic doses and clastogenic activity at toxic doses. Additional studies should primarily aim at determining the aneugenic hazard of trichlorfon to the somatic and germ cells of intact animals. The assessment of the risks associated with human exposure would require the availability of exposure data to allow the determination of the biological relevance of compound-related induced aneuploidy.

After the *in vitro* stage 1 testing of a pesticide, the assessor is presented with three potential outcomes, (i) positive response, (ii) negative response, (iii) equivocal response. Stage 2, a strategy for the *in vivo* testing of pesticides in germ cells, involves the evolution of the three responses in the somatic tissue of an intact rodent. In the case of a positive response the question is "whether the *in vitro* activity is also produced *in vivo*". There are several reasons why *in vitro* positives may not be reproducible *in vivo*, such as effective detoxification in an intact animal and the failure to reproduce conditions such as a high dose toxicity at target sites. Detailed consideration, including expert advice from the *in vitro* data at this stage, can result in a considerable saving in animal use and the failure to continue with the progress of a potentially valuable pesticide.

In the case of a negative response *in vitro*, the primary consideration is whether there is some deficiency in the *in vitro* assay, such as a lack of appropriate activating

enzymes that could lead to the failure to detect potential *in vivo* activity. In the case of a pesticide, there may be human exposure via operator use or via residues in the diet; then confirmation of negative mutagenic activity will require *in vivo* studies to confirm the lack of activity.

In the case of an equivocal response *in vitro*, it is generally unproductive to undertake additional *in vitro* studies if the data from the recommended package of *in vitro* tests are available. In such a case, *in vivo* testing is the most likely to produce clarifying data. However, the selection of an appropriate *in vivo* strategy should be based on expert judgment of the nature of the equivocal response. For example, if only weak equivocal results have been obtained in a bacterial gene mutation tester strain, then clarification of the response would not be helped by studying clastogenic activity in an intact rodent by, for example, using the rodent bone marrow micronucleus assay.

When the decision is made to test a pesticide for potential mutagenic activity *in vivo*, it should be influenced by the available *in vitro* database. In most cases, the *in vivo* test of first choice will be the bone marrow micronucleus assay of rodents. This test measures the induction of micronuclei (see earlier in the discussion of the *in vitro* assay) in the newly formed erythrocytes of the bone marrow, a tissue that is accessible to a wide range of chemicals. The induction of micronuclei in the rodent bone marrow indicates that a test chemical has aneugenic or clastogenic activity. These two activities can be identified by the use of kinetochore or centromere staining (see earlier) to distinguish between micronuclei containing whole chromosomes or fragments.

In the case of pesticides that are either negative or equivocal *in vitro*, a negative result in a rodent bone marrow micronucleus assay is supported by data confirming that the substance reached the tissue would provide reassurance of a lack of mutagenic activity. However, in the case of a pesticide with mutagenic activity *in vitro*, a negative result in a bone marrow micronucleus may not provide sufficient reassurance of a lack of activity *in vivo*, and information is required from another test system.

The selection of a second *in vivo* test system should be based upon expert consideration of a range of factors, such as the profile of the *in vitro* response(s), known as postulated metabolism and potential target tissues (eg, site-of-contact activity only).

Of the available second *in vivo* somatic assays, the choice of a method capable of detecting mutagenic end points is limited to cytogenetic (including micronucleus assays) in proliferating tissues, gene mutation assays using transgenic rodents such as Big Blue™ and Muta™ mouse [reviewed by Schmezer & Eckert (6)] and the analysis of mutation frequencies at specific sites within genes, such as restriction enzyme recognition

Table 3. Induction of the nondisjunction of chromosomes 2, 7, and 18 in a binucleate cell assay using cultured human lymphoblastoid cell line AHH-1 treated with the insecticide trichlorfon for 6 hours at pH 5.5. ^a

Concentration of trichlorfon	Binucleate cells (%)	Nondisjunction in 1000 binucleate cells			
		Chr 2	Chr 7	Chr 18	Total chromosomes 2 + 7 + 18
0 µg/ml	39.00	3	3	4	10
1 µg/ml	36.49	9	9	10	28
10 µg/ml	27.23	18 ^b	21 ^b	23 ^b	62
20 µg/ml	24.47	22 ^b	25 ^b	26 ^b	73
40 µg/ml	19.23	10 ^b	20 ^b	14 ^b	44
80 µg/ml	10.05	14 ^b	6 ^b	14 ^b	34

^a Data kindly supplied by Dr Anne Doherty.

^b P<0.05.

sites (7). In the case of the second *in vivo* assay, the choice has generally been extended to include indirect genotoxicity assays. They include methods which measure (i) DNA damage directly, such as the Comet assay and DNA unwinding, (ii) DNA adduct formation, such as P^{32} post-labeling, covalent binding, and mass spectrometer analysis for the quantification of modified nucleotides, and (iii) DNA repair activity, such as the rat liver unscheduled DNA synthesis (UDS) assay.

The rat liver UDS assay has been widely used as an indirect measure of potential mutagenic activity in an organ responsible for the metabolism of an extensive range of pesticides, and it is included in OECD guideline 486 (2).

In the cases in which negative results have been produced in somatic cell *in vivo* assays, it is generally believed that there is no need to proceed to stage 3, germ cell testing. At the time this report was written, no substance had yet been detected that produced negative results in somatic cells but unique positive results in germ cells *in vivo*. However, this situation may not continue as, on theoretical grounds, one might predict the existence of substances that interact with targets unique for germ cells, such as the pairing of chromosomes during the first division of germ cell meiosis. Currently, little information is available concerning the relative sensitivities of somatic and germ cells to genotoxic chemicals.

In the case of a newly developed pesticide for which comprehensive stage 1 and stage 2 negative data are available, it is highly unlikely that stage 3, the strategy for the *in vivo* testing of pesticides in germ cells, will be performed. However, for preexisting pesticides for which positive results have been obtained in somatic cells, there may be occasions when an estimate of germ cell mutagenic hazard may be required.

Currently, the assays for studying potential germ cell mutagenic activity are limited to male rodents and involve the analysis of (i) clastogenic effects in spermatogonial cells (OECD 483) (2), (ii) micronuclei in spermatocytes, (iii) aneuploidy in sperm using chromosome-specific molecular probes, (iv) dominant lethal effects in rodents after the mating of treated males with untreated females (OECD 478) (8), and (v) gene mutations in transgenic rodents.

Assays 1 to 5 provide evidence of mutagenic activity in male germ cells. Indirect evidence of DNA-interacting pesticides can be obtained using methods such as the P^{32} post-labeling assay for the quantification of DNA adduct formation. However, the demonstration of DNA adduct formation in male germ cells does not provide unambiguous evidence that the detected adducts will produce mutations in germ cells.

In cases in which human exposure has been shown to have occurred to a pesticide which is a germ cell mutagen, there may be a reason for providing an esti-

mate of the risks of such exposure. The risk assessment of germ cell mutations can currently be obtained by the application of two methodologies, the first being the specific locus assay, which involves the exposure of male mice to a test substance, mating to untreated females over the period of spermatogenesis and the quantification of mutations present in offspring. The assay provides a measure of the production of gene mutations for characters such as coat and eye color. The second is the heritable translocation assay, which measures the production of clastogenic effects by the detection and quantification of broken and rearranged chromosomes, which are transmitted to embryos. Both assays involve the use of large numbers of animals, and their use can only be justified in special circumstances.

Concluding remarks

The strategy of testing and the methodologies recommended provide the potential to provide a comprehensive profile of mutagenic activity for the determination of the hazard of pesticides and, when necessary, provide quantitative data for risk assessment. However, in cases in which mutagenic activity is detected, only at high and often toxic concentrations may the methodologies recommended in this report prove to be inadequate for the determination of biologically relevant no observable effects levels (NOEL) and potential threshold levels (9). The situation may also arise when a pesticide induces an increase in the mutation types observed in control animals.

To address the questions of hazard and risk assessment, an increasing range of methodologies is becoming available that make use of molecular techniques. Some examples include the use of an ever-increasing range of chromosome molecular probes with which to study and quantify chromosome structural and numerical changes (10) and the use of DNA sequencing to compare the types and positions of mutations in specific genes of control and treated animals. In the latter case, comprehensive databases have been developed that allow direct comparisons between spontaneous and compound-related mutation profiles. [See Lewis & Parry (11).]

Acknowledgments

The studies of the author were supported in part by funding from the European Union funded project, Protection of the European Population from Aneugenic Chemicals (PEPFAC), and the United Kingdom Food Standards Agency.

References

1. Committee on the Mutagenicity of Chemicals (COM). Guidance on a strategy for testing chemicals for mutagenicity. London: Department of Health; 2000.
2. Organisation for Economic Co-operation and Development (OECD). Ninth addendum to the OECD Guidelines for the Testing of Chemicals: update of section 4, health effects, revised guidelines. Paris: OECD; 1997. p 471, 473–6, 483, 486.
3. Fenech M, Morley AA. The measurement of micronuclei in lymphocytes. *Mutat Res* 1985;147:29–36.
4. Fenech M, Gott J, Turner J, Brown S. Necrosis, apoptosis, cytokinesis and DNA damage in human lymphocytes measured simultaneously with the cytokinesis-block micronucleus assay. *Mutagenesis* 1999;14:605–12.
5. Cziezel AE, Elek C, Grundy S, Metneki J, Nemes E, Reis A, et al. Environmental trichlofon and a cluster of congenital abnormalities. *Lancet* 1993;539–42.
6. Schmezer P, Eckert C. Induction of mutations in transgenic animal models: Big BlueTM and MutaTH Mouse. In: International Agency for Research on Cancer (IARC). The use of short and medium term tests for carcinogens and data on genetic effects in carcinogen hazard evaluation. Lyon: IARC; 1999. p 367–94. IARC publication, no 146.
7. Jenkins GJS, Suzen HS, Sueiro RA, Parry JM. The restriction site mutation assay: a review of methodology development and the current status of the techniques. *Mutagenesis* 1999;14: 439–48.
8. Organisation for Economic Co-operation and Development (OECD). Guidelines for testing chemicals. Paris: OECD; 1984. The Dominant Lethal Assay, no 478.
9. Parry JM, Fielder PJ, McDonald A. Thresholds for aneuploidy-inducing chemicals. *Mutagenesis* 1994;9:503–4.
10. Parry JM. Molecular cytogenetics. *Mutation Res* 1996; special issue 372:151–294.
11. Lewis PD, Parry JM. An explanatory analysis of multiple mutation spectra. *Mutation Res* 2002;518:163–80.