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Genetic Effects of Pesticides in the Mammalian Cells
I. Induction of Micronucleus

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농약이 포유동물세포에 미치는 유전적 영향
1. Micronucleus 유발

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적 요

Salmonella/microsome의 박테리아 시스템에서 돌연변이 유발성이 확인된 농약 중 살충제인 DDVP와 trichlorfon, 살균제인 TMTD, 제초제인 NIP와 MO 및 식물 생장조절제인 maleic hydrazide 등 6종의 화학물에 대한 포유동물세포에서의 유전적 영향을 마우스의 골수세포중의 polychromatic적혈구에 유발된 micronucleus를 조사함으로서 생체내에서의 염색체 이상 유무를 평가하였다.

조사한 농약중에서 오직 TMTD에서만 micronuclei의 출현빈도에 통계적인 유의성을 보였다. 우리생활에서 아주 많이 사용되는 유기인제 살충제인 DDVP는 micronuclei 출현을 증가시키지 않았으며 이외의 조사한 4종의 농약도 micronuclei 출현빈도에 영향을 주지 않았다.

INTRODUCTION

Pesticides are only a part of many new chemicals that enter our environment, but they are of particular concern because they are very potent biologically and are used so widely and in such enormous amounts (Epstein and Legator, 1971). A subtle risk from widespread use of pesticides lies in the possibility that some may be mutagenic or carcinogenic to man. Despite these possibilities, our information on their potential mutagenicity or carcinogenicity is greatly inadequate.

Some pesticides have been tested for mutagenicity in microbial systems and several of them have been found to be mutagenic in those test systems (Bridges, 1975; Kada *et al.*, 1974; Shirasu *et al.*, 1976; Wild, 1975). However, there is a question as to their relevance to man. It may be conceivable that some chemicals that give rise to positive results in microbial test systems would present a negligible hazard to man and *vice versa*.

A variety of techniques have been developed for the detection of chemical mutagens. Some of these techniques have recently been reviewed and compiled (Hollaender, 1971-1976). Our initial screening of pesticides for mutagenicity was done by the Ames' *Salmonella typhimurium* system which was rapid, sensitive, inexpensive and suitable for use in screening programs to evaluate the mutagenic activity of the large number of untested chemicals in our environment (Ames *et al.*, 1975). Using this system with recently introduced two new tester strains (TA 100 and TA 98) combined with an *in vitro* metabolic activation system, we were able to detect several pesticides of which mutagenicity had previously been escaped detection even from the extensive screening program by Shirasu *et al.*, (1976). They are insecticides, DDVP (dichlorvos), trichlorfon (Dipterex), Sumithion (fenitrothione) and naled (Dibrom), fungicide, TMTD, herbicides, NIP (TOK) and MO (see Byeon *et al.*, 1976).

Some of these compounds which have shown mutagenic activity in our bacterial system and an additional plant growth regulator maleic hydrazide which has been shown clastogenic in various plant cells were subjected to the further evaluation in the following mammalian test systems: (1) micronucleus test, (2) L5178Y *in vitro* mutagenesis test and (3) DNA repair assay system. In this paper only the results obtained by the micronucleus test were presented. The results from the other test systems will be published separately.

The micronucleus test developed by Schmid and coworkers is an *in vivo* cytogenetic sreening procedure for the detection of freshly induced structural and numerical chromosome aberrations in mammalian bone marrow cells (Boller and Schmid, 1970; Ledebur and Schmid, 1973; Matter and Schmid, 1971; Matter and Grauwiler, 1974). It is generally known that this test has numerous advantages over conventional metaphase analysis method in that scorable cells abound, the background level of aberrations is low and consistant, the preparative methods are simple and easy, and the test is independent of a favorable animal karyotype and highly suitable for revealing chromosome loss due to partial spindle disturbances (Schmid, 1975, 1976). Of the six pesticides tested, only TMTD exhibited the micronucleus inducing activity.

MATERIALS AND METHODS

The randomly-bred Swiss Webster albino mice aged 4~8 weeks were used. The test compounds were applied intraperitoneally (*i.p.*) in two or four daily doses. The compounds were dissolved in distilled water or dimethylsulfoxide (DMSO. final DMSO concentration was less than 5%) or suspended in vegetable oil. The tested dose range and lethal dose were shown in Table 1. Six pesticides; DDVP (dichlorvos, 2, 2-dichlorovinyl dimethyl phosphate), trichlorfon (Dipterex, 0, 0-dimethyl (2, 2, 2-trichloro-1-hydroxyethyl) phosphonate), TMTD (thiram, bis (dimethyl-thiocarbamoyl) disulfide), MO (2, 4, 6-trichlorophenyl-4-nitro-phenyl-ether), NIP (TOK, 2, 4-dichlorophenyl-*p*-nitro-phenylether) and maleic hydrazide (MH-30) used in the experiments were pure or technical grade. They were obtained from the Korean National Institute of Health and the Korean National Agricultural Material Inspection Office. An alkylating cytostatic cyclophosphamide (Endoxan, Asta) was employed as a positive control.

The mice were sacrificed by cervical dislocation 6 hours after the last dose. The bone marrow from both femora was harvested using fetal calf serum (Difco) and prepared for microscopic examination according to the procedure of Schmid (1973). Three smeared slides were prepared for each mouse and air-dried for over an hour, and then were stained with Wright-Giemsa stain. The slides were coded and examined under the microscope at a magnification of 1,500 ×. About 2,000 polychromatic erythrocytes per mouse were analysed for the presence of micronuclei. In this paper the frequencies of micronucleated cells within polychromatic erythrocytes are given as the per thousand (‰). The standard tables of Kastenbaum and Bowman (1970) were used for the statistical analysis tests of the significance for differences in the frequency of micronuclei of the each treated from the control group.

RESULTS

A toxicity test was performed for each pesticide to determine the concentration at which animals could be exposed to maximum dose (Table 1). The acute toxicity tests showed that organophosphorus insecticides, DDVP and trichlorfon and organosulfur fungicide TMTD were extremely toxic to mice. And the other pesticides tested had moderate toxicity. In this study, the test animals were to receive each compound up to the maximum non-lethal dose.

Micronuclei in erythrocytes have been known routinely as Howell-Jolly bodies by the hematologists. Micronuclei in polychromatic erythrocytes are round with a

Table 1. Summary of results from toxicity test and micronucleus test of pesticides in mice. IP treatment, 2 doses separated by 24 hours.

Pesticides	Class[a]	Solvent[b]	Lethal dose(mg/kg)	Dose range tested(mg/kg/d)	MNT results
DDVP	I	H_2O	0.03	0.0075 –0.015	–
Trichlorfon	I	H_2O	625	125 –312.5	–
TMTD	F	DMSO	200	12.5 –100	+
MO	H	DMSO	4,000	500 –1,000	–
NIP (TOK)	H	DMSO	4,000	500 –1,000	–
Maleic hydrazide	G	Vegetable oil	1,200	100 –600	–

a. F, Fungicide; I, Insecticide; H, Herbicide; G, Growth regulator.
b. In case of chemicals that were suspended in DMSO, the final dimethylsulfoxide concentration was less than 5%.

diameter of about 1/20 to 1/5 of an erythrocyte. However, there is a little variation in morphology of micronuclei. Fig. 1 shows the various shapes of micronuclei in polychromatic erythrocytes induced by a positive control drug,cyclophosphamide. Micronuclei in leucocytes are also shown in Fig. 1. However, they are not evaluated quantitatively in this assay because they are difficult to distinguish

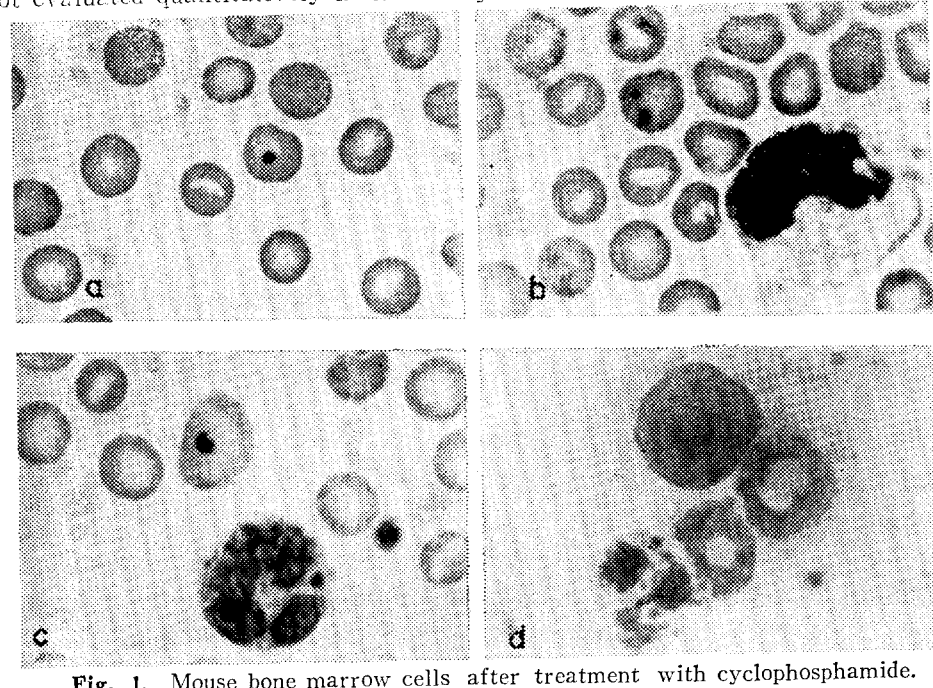

Fig. 1. Mouse bone marrow cells after treatment with cyclophosphamide. (a–b) Typical morphology of micronuclei in polychromatic erythrocytes. (c) Almond shaped micronucleus. (d) Micronuclei in leucocytes. They are difficult to distinguish from normal nuclear lobes or projections.

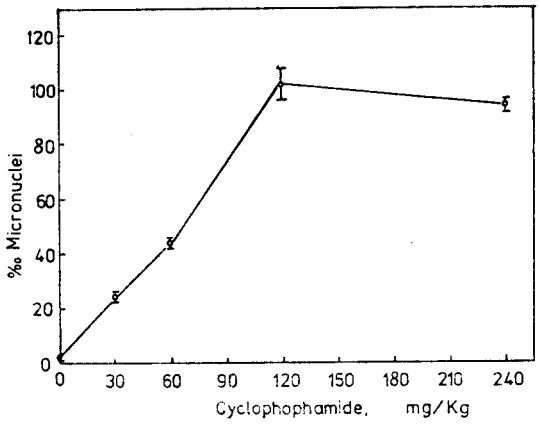

Figure 2. Dose-effect curve obtained with cyclophosphamide.

from normal nuclear lobes or projections.

The dose-effect relationship obtained with cyclophosphamide is illustrated in Fig. 2. The result is similar to those of other workers (Goetz *et al.*, 1975; Maier and Schmid, 1976) except for the plateau at the higher doses.

Table 2. Results of the micronucleus test in mice after subacute intraperitoneal treatment with pesticides. 2 doses separated by 24 hours.

Pesticides	Single dose (mg/kg)	No. of mice	No. of micronucleated cells analysed	Micronuclei ‰ (mean ± S.E.)
DDVP	0.015	3	33/6700	4.93±0.35
Trichlorfon	125	3	22/6600	3.33±0.77
	312.5	3	30/5400	5.56±0.43
TMTD	12.5	3	23/5100	4.51±0.11
	25	3	44/8400	5.24±0.62
	50	3	49/6000	8.17±1.06[a]
	100	4	102/8300	12.29±0.69[a]
MO	500	3	19/8200	2.32±0.31
	1000	3	19/7600	2.50±0.62
NIP (TOK)	500	3	15/6500	2.31±0.18
	1000	3	14/5900	2.37±0.62
Maleic hydrazide	100	3	39/6900	5.65±0.61
	600	3	11/4500	2.44±0.29
Cyclophosphamide[b]	30	3	211/8600	24.53±2.08[a]
	60	3	339/7700	44.03±1.04[a]
	120	3	690/6750	102.22±6.13[a]
	240	3	496/5300	93.58±2.74[a]
Control (pooled)				3.08±0.23

a: P. significantly higher than 0.05. Tables of Kastenbaum and Bowman (1970) used for determining statistical significance.

b: Cyclophosphamide was used as a positive control.

The results of micronucleus test performed with the pesticides are summarized in Table 2. Frequencies of micronuclei in individual controls vary between 0~5 per thousand (the mean value for all experiments is about 3 per thousand). Among the pesticides tested only TMTD shows a significant difference from controls at the 5% confidence level by the table of Kastenbaum and Bowman (1970). There is a moderate dose-response among the TMTD treatments. Fig. 3 shows the relative increase of micronuclei by TMTD over control value.

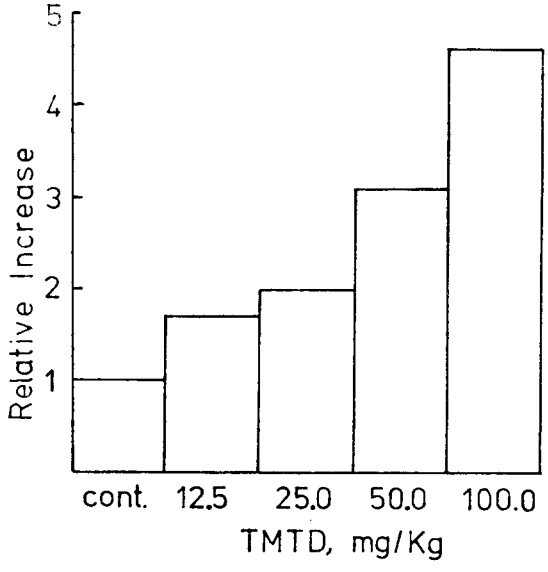

Fig. 3. TMTD induced relative increase of micronuclei over control.

Organophosphorus insecticide DDVP which is the most broadly used and economically important chemical, did not increase the micronuclei frequencies after two or four daily injection (Table 3).

Table 3. Incidence of micronuclei after 2 or 4 treatments with DDVP

Duration of drug treatment	Single dose (μg/kg)	No. of animals	No. of micronucleated cells analysed	Micronuclei ‰ (mean±S.E.)
2 days	Control (saline)	3	22/6400	3.44±0.22
	15	3	33/6700	4.93±0.35
4 days	Control (saline)	3	37/7750	4.77±0.14
	7.5	4	38/8300	4.58±0.22
	15	4	57/9400	6.06±0.31

DISCUSSIONS

Insecticides are in general very potent biologically. A particular subtle danger from wide scale use of pesticides lies in the possibility that some may be mutagenic, carcinogenic or teratogenic to man. Because of the close correlation between mutagenic and carcinogenic, teratogenic and other deleterious biological activities of chemicals, it is indispensable to test scrutinizingly the mutagenicity of insecticides. Hence potential genetic effects of selected insecticides were investigated with various assay systems (see Wild, 1975).

Many pesticides in widespread use are known as mutagens in experimental organisms. Most of the results were obtained with sub-mammalian system such as bacteria, fungi or *Drosophila*. However, very few of them have been tested in mammalian system for the potential mutagenic hazard to man. Bridges (1974) intensely proposed that the compounds likely to be exposed to the general population must be tested for mutagenicity by tests involving mammals. The committee 17 Report (1975) also recommended that the compounds that are proposed for actual distribution should be subject to mammalian screening tests. Therefore present study should contribute to extrapolate the bacterial mutagenicity data to the problems of human health hazards. We have shown that DDVP, trichlorfon, TMTD and NIP induce base-substitution mutations and MO frame-shift mutation in the *Salmonella*/rat liver microsome system. Among these pesticides only organosulfur fungicide TMTD induced the micronuclei in the mouse bone marrow cells. The dose-effect relationships could be demonstrated for this compound (Figure 3).

Shirasu and his associates showed that organosulfur fungicides, TMTD, ziram (Zinc dimethyldithiocarbamate), Bis-dithane [dizinc bis-(dimethyldithiocarbamate) ethylene-bis (dithiocarbamate)], and ETM [N,N′-ethylene-bis (thiocarbamoyl) sulfide] were positive in the rec-assay, but only TMTD was positive in the *S. typhymurium* (Shirasu, 1976). Using the microbial systems, on the one hand, Warren *et al.*, (1976) tested the mutagenicity of dithiocarbamate and thiocarbamoyl disulfide fungicides and then showed thiram (TMTD) was only lethal to *exr*$^-$ mutants of *E. coli*. To our knowledge, however, no assays for mutagenicity in mammalian systems have been published so far. In order to examine further cytogenetic effects of the TMTD, the metaphase analysis of the mouse bone marrow cells *in vivo* and of short-term cultured human peripheral lymphocytes *in vitro* are now in progress. Although several types of mutagenesis experiments have been performed with organophosphorus insecticide DDVP in cultured mammalian cells or in experimental mammals, it has failed to detect any mutagenic effects of DDVP in most tests (Dean, 1972; Dean and Thorpe, 1972a; 1972b). Our results of the micronucleus test (Table 3) showed that DDVP did not increase the micronucleus incidencies in

mouse bone marrow cells after two or four daily injection.

Another organophosphorus insecticide trichlorfon also failed to increase the micronuclei frequencies in mice. Benes and Sram(1969) reported that trichlorfon did not induce the sex-linked recessive lethal mutation in *Drosophila*. From the chromosomal studies in patients suffering acute organophosphate insecticide intoxication, Van Bao *et al.* (1974) reported that in the malathion intoxicated group a significant increase of chromatid and chromosome type aberrations immediately after the exposure was seen. They also showed that in the trichlorfon group no immediate change was found. Therefore, it may be concluded that trichlorfon do not have any clastogenic effect in mammals.

It has been known that maleic hydrazide caused chromosome aberrations in various plant cells. But a negative result was reported from the dominant lethal test in mice (Epstein and Legator, 1971). In our study, this plant growth regulator did not increase the frequency of micronuclei in mice (Table 2). Herbicides, Mo and NIP also gave negative responses in micronucleus test system.

In order to make quantitative estimation of the mutation risk to man, considerable information on the genetic effects of pesticides has to be accumulated from the various test organisms and systems. The results of the micronucleus test in mice would be useful information in this respect.

SUMMARY

In order to evaluate the mutagenic potential in animal for those pesticides which were proved to be mutagenic in the bacterial screening system with a metabolic activation *in vitro*, we have studied *in vivo* cytogenetic effects on mouse bone marrow by means of the micronucleus test. The clastogenic activity of the chemical is evaluated as the frequency of micronuclei in polychromatic erythrocytes.

We have tested six pesticides, insecticides, DDVP and trichlorfon, fungicide, TMTD, herbicides, NIP and MO and growth regulator, maleic hydrazide. It was found that among the tested pesticides only TMTD exhibited minimal activity in inducing micronuclei. Organophosphorus insecticide DDVP that is the most broadly used and economically important chemical, did not increase the micronuclei frequencies in mouse bone marrow cells as with the all other pesticides tested.

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REFERENCES

Ames, B.N., J. McCann and E. Yamasaki, 1975. Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian microsome mutagenicity test. *Mutation Res.* **31** : 347—364.

Benes, V. and R. Sram, 1969. Mutagenic activity of some pesticides in *Drosophila melanogaster*. *Ind. Med. Surg.*, **38** : 50—52.

Boller, K. and W. Schmid, 1970. Chemische Mutageneses beim Säuger. Das Knochenmark des Chinesischen Hamsters als in vivo-Testsystem. Hämatologische Befunde nach Behandlung mit Trenimon. *Humangenetik* **11** : 35—54.

Bridges, B.A., 1974. The three-tier approach to mutagenicity screening and the concept of radiation equivalent dose. *Mutation Res.* **26** : 335—340.

Bridges, B.A., 1975. The mutagenicity of captan and related fungicides. *Mutation Res.* **32** : 3—34.

Byeon, W.H., H.H. Hyun and S.Y. Lee, 1976. Mutagenicity of pesticides in the *Salmonella* /microsome system. *Kor. Jour. Microbiol.* **14** : 128—134. (in Korean).

Committee 17 of the Council of the Environmental Mutagen Society, 1975. Environmental mutagenic hazards. *Science* **187** : 503—514.

Dean, B.J., 1972. The effect of dichlorvos on cultured human lymphocytes. *Arch. Toxikol.* **30** : 75—85.

Dean, B.J. and E. Thorpe, 1972 a. Cytogenetic studies with dichlorvos in mice and Chinese hamsters. *Arch. Toxikol.* **30** : 39—49.

Dean, B.J. and E. Thorpe, 1972 b. Studies with dichlorvos vapor in dominant lethal mutation tests on mice. *Arch. Toxikol.* **30** : 51—59.

Epstein, S.S. and M.S. Legator, 1971. The mutagenicity of pesticides. Concepts and Evaluation. The MIT Press, Cambridege, Massachusetts, *pp.* 220.

Goetz, P., R.J. Sram and J. Dohnalova, 1975. Relationship between experimental results in mammals and man. *Mutation Res.* **31** : 247—254.

Hollaender, A. (Ed.) Chemical Mutagens, Principles and Methods for their Detection, Plenum Press New York, Vols. **1, 2,** 1971; Vol. **3,** 1973; Vol. **4,** 1976.

Kada, T., M. Moriya and Y. Shirasu, 1974. Screening of pesticides for DNA interactions by "Rec-Assay" and mutagenesis testing and frameshift mutagens detected. *Mutation Res.* **26** : 243—248.

Kastenbaum, M.A. and K.O. Bowman, 1970. Tables for determining the statistical significance of mutation frequencies. *Mutation Res.* **9** : 527—549.

Ledebur, M. von and W. Schmid, 1973. The micronucleus test. Methodological aspects. *Mutation Res.* **19** : 109—117.

Maier, P. and W. Schmid, 1976. The model mutagens evaluated by the micronucleus test. *Mutation Res.* **40** : 325—338.

Matter, B.E. and J. Grauwiler, 1974. Micronuclei in mouse bone-marrow cells. A simple *in vivo* model for the evaluation of drug induced chromosomal aberrations. *Mutation Res.* **23** : 239—249.

Matter, B.E. and W. Schmid, 1971. Trenimon-induced chromosomal damage in bone-marrow cells of six mammalian species, evaluated by the micronucleus test. *Mutation Res.* **12** : 417—425.

Schmid, W., 1973. Chemical mutagen testing on *in vivo* somatic mammalian cells. *Agents and Actions* **3** : 77—85.

Schmid, W., 1975. The micronucleus test. *Mutation Res.* **31** : 9—15.

Schmid, W., 1976. The micronucleus test for cytogenetic analysis. *In* Chemical Mutagens (A. Hollaender, editor). Plenum Pub. Co., New York **4** : 31—53.

Shirasu, Y., 1976. Mutagenicity screening on Pesticides. *In* Laboratory Manual for East-Asian Workshop on Mutagenicity Testing of Chemicals. pp. 69—72. Mishima, Japan.

Shirasu, Y., M. Moriya, K. Kato, A. Furuhashi and T. Kada, 1976. Mutagenicity screening of pesticides in the microbial system. *Mutation Res.* **40** : 19—30.

Van Bao, T., I. Szabo, P. Ruzicska and A. Czeizel, 1974. Chromosome aberrations in patients suffering acute organic phosphate insecticide intoxication. *Humangenetik* **24** : 33—57.

Warren, G., P.D. Skaar and S.J. Rogers, 1976. Genetic activity of dithiocarbamate and thiocarbamoyl disulfide fungicides in *Saccharomyces cerevisiae, Salmonella typhimurium* and *Escherichia coli*. *Mutation Res.* **38** : 391—392 (*Abstract*).

Wild, D., 1975. Mutagenicity studies on organophosphorus insecticides. *Mutation Res.* **32** : 133—150.