KOREAN JOURNAL OF ZOOLOGY
Vol. 20, No. 4, 159~168 (1977)

Genetic Effects of Pesticides in the Mammalian Cells
Ⅱ. Mutagenesis in L5178Y Cells and DNA Repair Induction

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농약이 포유동물세포에 미치는 유전적 영향
Ⅱ. L5178Y 세포의 돌연변이 유발성과 DNA회복합성유발

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(Received October 25, 1977)

적 요

계대 배양중인 생쥐의 임파종양 L5178Y 세포의 유전자돌연변이 유발성 검출법 (Methotrexate-저항성)과 순수 분리한 사람의 임파구에서의 DNA 회복복제법을 사용하여 *Salmonella*/microsome 시스템에서 돌연변이 유발성이 확인된 살충제 DDVP와 trichlorfon, 살균제 TMTD 및 제초제 MO와 NIP등 5종의 농약이 포유동물 세포에 미치는 유전적 영향을 조사했다.

조사한 농약중 TMTD는 상기조사한 시스템 모두에서 양성결과를 보여준 반면에 DDVP와 trichlofon은 L5178Y 세포에서의 돌연변이 유발성은 나타내지 않았으나 DNA회복 복제법에서는 양성결과를 보여주었다. MO와 NIP는 조사한 시스템 모두에서 양성결과를 나타내지않았다.

INTRODUCTION

Advances in science and technology have led to the introduction of extraordinarily large amounts of already naturally occurring chemicals and to millions of new and foreign substances, most of whose biological effects are unknown and either untested or only incompletely tested. A particular subtle danger from exposure to these chemicals lies in the possibility that some may be mutagenic.

The availability of mammalian cell culture system let us no longer have to rely on microorganisms to determine mutagenicity (Puck, 1972). Of the mammalian methods that have been proposed, the murine lymphoma cell line L5178Y, which grows rapidly both in suspension culture and in the peritoneal cavity of genetically compatible mice (DBA/2 or BDF_1) provides a useful method of detection for

mutagenicity(Clive *et al.*, 1973 ; Cole and Arlett, 1976 ; Fischer *et al.*, 1974 ; Lee, 1973 ; Knaap and Simons, 1975 ; Nakamura *et al.*, 1977).

A number of known mutagens and carcinogens have been evaluated for their abilities to induce unscheduled DNA repair synthesis in cultured mammalian cells (Clarkson and Evans, 1972 ; Legator and Flamm, 1973 ; Lieberman *et al.*, 1971 ; Regan and Setlow, 1973). It is anticipated that this method could identify genetically active compounds that induce DNA damage. The assay system, developed by Lieberman *et al.* (1971), that utilizes the non-proliferative peripheral lymphocytes and liquid scintillation counting method to detect unscheduled DNA synthesis is simple, speedy and relevant to problems of human exposure which are requirements of screening method for possible mutagens.

We have attempted to make comparative analyses with the various assay systems to evaluate the mutagenic risk of pesticides in human beings. Some results that were obtained from the bacterial screening system using the Ames *Salmonella typhimurium* and from the micronucleus test in mouse bone marrow were reported previously(Byeon *et al.*, 1976 ; Paik and Lee 1977). In this paper the results obtained from the L5178Y cells *in vitro* mutagenesis test and DNA repair assay are presented.

MATERIALS AND METHODS

1. *Test Chemicals*

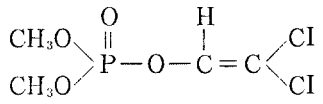
Organophosphorus insecticides DDVP and trichlorfon organosulfur fungicide TMTD and two herbicides, MO and NIP were investigated in this study. They were pure or technical grade and were obtained from the Korean National Institute of Health or the Korean National Agricultural Material Inspection Office. The chemical structures of these compounds are shown in Fig. 1. DDVP was dissolved in sterilized distilled water. And all the other pesticides were dissolved in sterilized dimethylsulfoxide (DMSO) and subsequently diluted with culture medium to the required concentration. The final DMSO concentration was less than 1%.

Ethylmethanesulfonate (EMS, K & K Laboratories, Plainview, N.Y.), a potent monofunctional alkylating mutagen, was used as a positive control in mutagenesis studies. A nitrogen mustard, nitromin (methyl bis (β-chloroethyl)-amine-N-oxide hydrochloride, Yoshitomi, Japan) was used in the DNA repair assay as a reference.

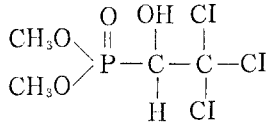
2. *Mutagenicity Test*

Cells

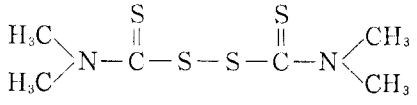
The subclones of L5178Y were selected from the parent murine leukemic cell line for mutagenesis studies. They form round, dense, tridimensional uniform



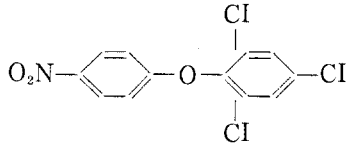
DDVP (Dichlorvos)
2, 2-dichlorovinyl dimethyl phosphate
M.W=220.98



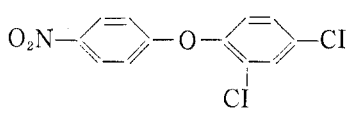
TRICHLORFON (Dipterex®)
0. 0-dimethyl(2.2.2-trichloro-1-hydroxyethyl) phosphonate
M.W=257.44



THIRAM (TMTD)
Bis(dimethyl thiocarbamoyl) disultide
M.W=240.44



MO
2, 4, 9-trichlorophenyl-4-nitro-phenylether
M.W=319.6



NIP (TOK)
2, 4-dichlorophenylp-nitro-phenylether
M.W=284.1

Fig. 1. Chemical structures and names of pesticides.

colonies in soft agar medium (0.12%) with a high cloning efficiency (over 80%). They grow rapidly in suspension culture (10—12 hour doubling time) to high cell densities and retain a near diploid of 40 acrocentric chromosomes. Stock cultures of the cells were maintained in the exponential phase of growth in Fischer's medium (Grand Island Biological Company, N.Y.) supplemented with 10% horse serum (Home made) and antibiotics penicillin and streptomycin (FMS). Stock cells were also maintained at -70°C according to a method previously described elsewhere.

The culture techniques were employed according to the method of Fischer and Sartorelli (1964). Cells were counted in Coulter Particle Counter (Model A, Coulter Electronics).

Cytotoxicity

The viability of cells *in vitro* was determined by the soft-agar cloning method (Fischer, 1973).

Mutagenesis

Samples of 2×10^6 cells in 10ml of FMS were exposed in duplicate to the test chemicals for 4 hr or for 18 hr at a dosage which produces approximately 80% cell kill and at a half dose of that drug level. After removal of the chemical the cells were incubated in growth medium (FMS) at 37°C for 96 hr to permit expression of the mutant. Control cells, in parallel with the treated cells, were maintained in the exponential phase of growth. Among the number of genetic markers available in L5178Y cells, a drug resis tance marker, methotrexate (MTX)

was used at 6×10^{-8}M. Mutants were recovered by the soft-agar cloning method. Quadruplicate tubes were set up with 4×10^{5} cells per tube for each drug level used. In order to determine cloning efficiency, 80 cells per tube were placed in soft-agar medium free of the selective agent. The number of mutants were normalized to 100% cloning efficiency. The experiment was repeated at least three times in order to avoid the occasional fluctuations of mutant frequencies which might occur in different cell populations, thus resulting in false positives. The mutant frequency was calculated by normalized number of mutants per total number of cells added.

3. *DNA Repair Assay*

Cells

Human peripheral blood lymphocytes from healthy men were prepared by the Ficoll-Hypaque gradient centrifugation (Yi and Kim, 1975). The purified lymphocytes (1×10^{6} cell/ml) were cultured in 2 ml of FMS.

Induction of cell damage and use of hydroxyurea

Cells were exposed to chemicals for 1 hr in Fisher's medium without horse serum. After than, cultures were centrifuged and the medium was replaced with fresh medium containing 10 mM hydroxyurea (HU, K & K Laboratories, Plainview, N.Y.) in order to suppress the normal semi-conservative DNA replication. Control cultures were handled in the same manner as the test cultures except for avoiding the test chemicals.

Liquid scintillation counting

After damaging cells were cultured with a final concentration of 0.5 μCi/ml tritiated thymidine (^{3}H-TdR, 45 Ci/mmol, Amersham-Searle Co., England) in the presence of 10 mM HU for 3 hr.

The reaction was stopped by cooling the culture tubes in ice, followed by centrifuging at 800 g for 10 min at 4°C and aspirating off the radioactive medium. The cells were washed once with ice-cold 0.9% saline solution and precipitated with ice-cold 10 % Trichloroacetic acid (TCA). The precipitates were collected on glass fiber filters (Reeve Angle), washed with 1% and then washed with 0.1 % TCA, dried, and placed in toluene scintillation mix (4g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis [2-(5-phenyloxazolyl)] benzene per liter of toluene). They were counted in a liquid scintillation spectrometer (Nucelar-Chicago). Each experiment was performed at least twice.

RESULTS

Mutagnicity test with L5178Y cells

The cytotoxicity tests were first performed in order to determine adequate drug doses for the mutagenesis assay (Fig.2). It was found that TMTD was the

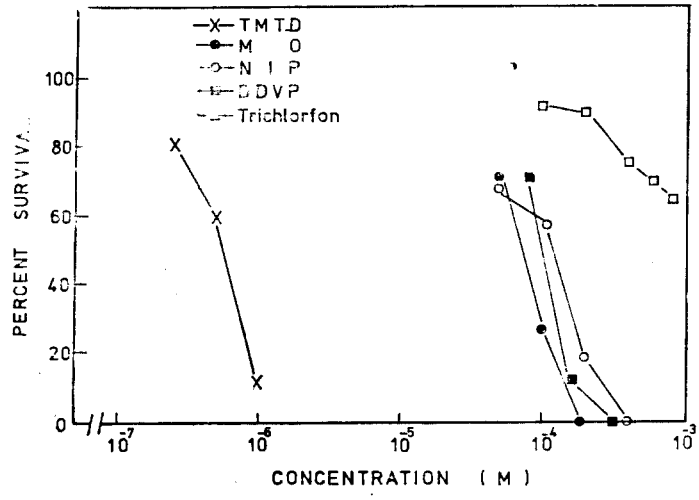


Fig. 2. Survival curves of L5178Y cels as a function of pesticides.
5×10^4 L5178Y cells were exposed to the chemical for 4 hr (DDVP and Trichlorfon) or for 18 hr (TMTD, MO and NIP). After exposure to chemical, the cells were resuspended in chemical-free FMS. Then the cell survival was determined by soft-agar cloning method. On day 10 after cloning, the survived colonies were counted. Each point represents the arithmetic mean of two experiments.

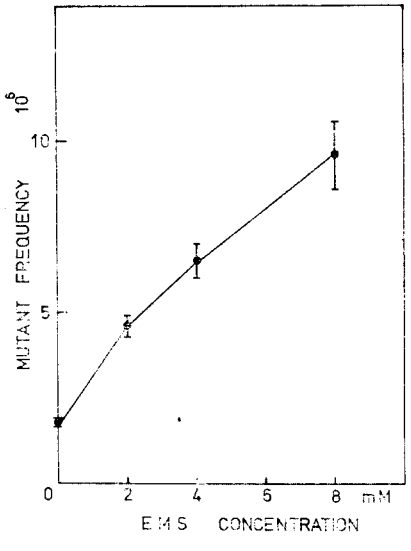


Fig. 3. *In vitro* induction of MTX-resistants by various concentrations of EMS in L5178Y cells.
10^6 cells in 10 ml of FMS were exposed in duplicate to the EMS concentrations as indicated for 1 hr at 37°C. To permit expression of mutants, cells were then incubated for 96 hr in the EMS-free medium before cloning. MTX was used as the selective agent at 6×10^{-8}M. Quadruplicate tubes were set up with 4×10^5 cells/tube for each drug level used. The number of mutants were normalized to 100% cloning efficiency.

most toxic and trichlorfon was the least toxic to L5178Y cells *in vitro*.

Fig.3 shows an example of positive mutagenic response obtained from the MTX-mutagenesis assay in L5178Y cells induced by EMS. The mutagenicity results from pesticides are summarized in Table 1. A significant increase in mutant frequency was appearent in the treated cells *in vitro* with 5.0×10^{-7}M TMTD for 18 hr. We could not, however, establish complete dose-response relationships with this pesticide due to the toxicity at higher concentrations. Organophosphorus insecticides DDVP and trichlorfon and herbicides MO and NIP were all negative in this test system.

DNA repair assay

Using purified human peripheral lymphocytes DNA repair assay system developed by Lieberman *et al.* (1971),

Table 1. Induction of MTX-resistant mutants of L5178Y cells treated *in vitro* with pesticides

Compound	Treated hours	Concentration (M)	Mutation frequency per survivor($\times 10^{-6}$)	MF_t/MF_c[a]
DDVP	4	0	3.75	
		8×10^{-5}	4.89	1.3
		16×10^{-5}	4.83	1.3
Trichlorfon	4	0	3.93	
		2×10^{-4}	3.13	0.8
		4×10^{-4}	5.65	1.4
TMTD	18	0	2.50	
		2.5×10^{-7}	3.44	1.4
		5.0×10^{-7}	9.25	3.7[b]
MO	18	0	3.92	
		5×10^{-5}	4.38	1.1
		10×10^{-5}	4.34	1.1
NIP	18	0	3.92	
		1×10^{-4}	4.25	1.1
		2×10^{-4}	4.19	1.1

For the experimental conditions, see legend of Fig. 3 and details in the text.

(*a*) Ratio of the mutant frequency of treated cells to that of control cells.

(*b*) $P<0.05$. Tables of Kastenbaum and Bowman (1970) were used for determining statistical significance.

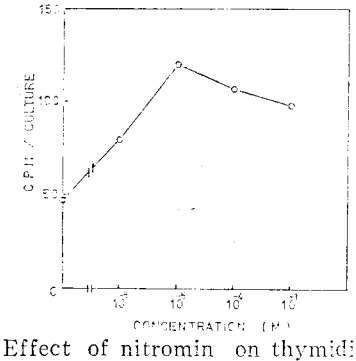


Fig. 4. Effect of nitromin on thymidine incorporation by lymphocyte cultures.

Cells (2×10^6 per culture) were exposed to the indicated doses of nitromin for 1 hr and then labeled with a concentration of 0.5μCi/ml ^{3}H-TdR in the presence of 10 mM HU for 3 hr. The acid-insoluble counts were measured.

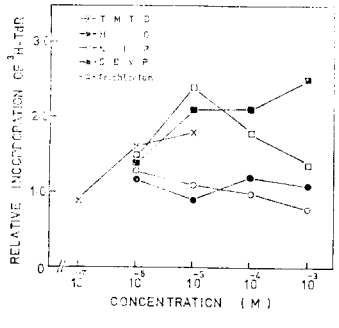


Fig. 5. Effectof pesticides on thymidine incorporation by lymphocytes.

See legend of Fig. 4. Values are relative to nontreated control incorporation.

it could be obtained an assured unscheduled DNA repair synthesis against nitromin damages. Fig.4 shows nitromin induced dose-response curve of ^{3}H-thymidine incorporation in human lyphocytes *in vitro*. Five pesticides were tested for DNA repair indnction with this assay system. The results obtained are shown in Fig. 5.

^{3}H-thymidine incorporation in the DDVP treated cells was increased about 2—2.5 times over the untreated control. A similar result was obtained with trichlorfon but the incorporation was declined at the higher concentrations TMTD also slightly increased incorporation of the labeled precursor. However, no significant increases were observed with the tested concentrations of NIP and MO

DISCUSSION

With increasing use of pesticides in agriculture it is inevitable that traces or residues of these chemicals enter our environment(Epstein and Legator,1971). The important biologibal risks associated with exposure to these chemicals are the induction of mutations in germ cells and of cancers in somatic cells. We can not afford a sudden rise in genetic or cancer risks in our population on the one hand, but on the other hand do not we afford to ban potentially bencefitial pesticides simply because they respond possitively to a microbial test system. In order to balance benefit against risk, it is essential to use systems that have a high degree of presumptive human relevance (Committee 17, 1975). This study is a part of our effort that comparative analyses be made with the various mammalian systems.

All the tested pesticides in this study were shown mutagenic activities in bacterial system (Byeon *et al.*, 1976). However, only TMTD among othse mutagenic pesticides in bacteria was previously reported for a slight clastogenicity(Paik and Lee, 1977). In this study, it was found that TMTD was weakly mutagenic in L5178Ycells *in vitro* in MTX-mutagenesis test system and also that DDVP, trichlorfon and TMTD induced unscheduled DNA repair synthesis in human lymphocytes.

Organophosphrus insecticide DDVP was in general mutagenic to nearly all the bacterial species tested (Byeon *et al.*, 1976 ; Dean, 1972a; Voogd *et al.*, 1972). However, a mutagenic action of DDVP could not be established in the host-mediated assay (Voogd *et al.*, 1972). And it also failed to detect any mutagenic effects of DDVP in most mammalian test systems(Dean 1972b; Dean and Thorpe, 1972, a, b). We also were unable to detect a mutagenic effect of DDVP in the L5178Y cells mutagenes system *in vitro*. The results certainly indicate that rapid catablism(metabolic degradation) in the mammal or in the protein-rich culture media for mammalian cells greatly reduce a potential DDVP effect. However, Wyrobek and Bruce(1975) reported that DDVP induced slightly elevated levels of sperm abnormalities in mice. Wild(1975) reviewed the effect of organophosphorus insecticides on cellular DNA. It was reported that DDVP and trichlorfon reacted with DNA in bacteria (Wild, 1975). In our study, it was observed that unscheduled DNA synthesis was slightly induced by DDVP and also by trichlorfon in

cultured human lymphocytes.

Organosulfur fungicide TMTD was positive both in the rec — assay and in the *S. typhimurium* mutagenesis studies (Byeon *et al.*, 1976 ; Kada *et al.*, 1974). From this and our previous studies, we know that TMTD responded positively to all three mammalian systems tested. TMTD associated with these apparent mutaegnic effects on mammalian cells must be re-evaluated because of its practical importance and its elevated residues in the environment. Although MO and NIP were found to be mutagenic in the microbial test system, they gave all negative responses in our mammalian test systems.

With the development of genetic toxicology, it is quite conceivable that evaluating environmental agents for mutagenicity will be of greater significance to the well-being of the human population than the evaluation of these chemicals for any other known toxic effect. In this respect, the results obtained from these mammalian mutagenesis studies would be useful.

SUMMARY

In order to evaluate the mutagenic potentential in mammalian system for those pesticides which were proved to be mutagenic in *Salmonella* microsome assay system, we have studied drug-resistant mutagenesis in cultured L5178Y cells and unscheduled DNA synthesis in human lymphocytes *in vitro*. We have tested five pesticides: insecticides DDVP and trichlorfon, fungicide TMTD and herbicides MO and NIP.

Of these pesticides, TMTD induced weak mutation to MTX-resistance in L5178Y cells *in vitro* and gave positive responses in DNA repair assay system. Therefore, its potential genetic risks in human beings should be re-evaluated.

DDVP and trichlorfon gave negative response in L5178Y mutagenesis test system but stimulated incorporation of ^{3}H-TdR in DNA repar assay. MO and NIP gave also negative responses both in L5178Y mutagenesis test systemand in DNA repair assay system.

ACKNOWLEDGMENTS

The anthors wish to thank Mrs. S.D. Hong(Lee) for her excellent technical assistance. This investigation was snpported in part, by the International Atomic Energy Agency under the contract number 1637/R1/RB.

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