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Pesticide Induced Cytogenetic Risk Assessment in Human Lymphocyte Culture *In Vitro*

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Pesticides have wide spectrum of activity in controlling pests, differing greatly in their mode of action and toxicity to humans (Woo *et al* 1996; Degreave et al 1979). Potential exposure of pesticides from the environment can be estimated by environmental monitoring and actual exposure can be measured by the biological monitoring of human tissues and body fluids. Pesticides and metals have major role in humans, plants and insects (Kaiser Jamil 1992). Genotoxicity is an important hallmark of various genetic diseases, hence there is a need to understand the type of damage, a chemical or a pesticide can make on the DNA. This understanding may be helpful in comprehending the susceptibility to various genotoxic agents. Since toxicity depends on the structure of chemicals, quantitative structure activity relationship (QSAR) becomes an important aspect of biological studies (Perkins *et al* 2003). As we enter the new millennium, we are focusing our attention on in-vitro technologies, which can be directly related to humans, instead of extrapolating the data from animal model studies. Biomarkers are now widely used to detect the effects of pesticides before adverse clinical health effects occur (Waters 1980; Yoder1973). The biomarkers which are known in genetic toxicology are generally determined by using several assays like micronucleus assay, chromosomal aberration frequencies, sister chromatid exchanges, and more recently comet assay or single cell gel electrophoresis (Saleha Banu etal 2001)

Pesticide toxicity has been extensively investigated; several reports (Amer 1992; Klopman 1985; Nelson 1990) deal with genotoxic effects of commonly used pesticides. However, only few reports on the cytogenetic and genotoxic effects of these pesticides in the invitro systems using lymphocytes from peripheral blood of humans are available (Ford 1998; Lander 2000; Davor Zetjezic 2001). International program on chemical safety, health and safety guide no.80 (Extension toxicology network) reports the toxicity of Monocrotophos (0.006mg/kg), Chlorpyriphos (0.01mg/kg), Dimethoate (2.0mg/day) and Endosulfan (0.006 mg/kg) as non-toxic to humans, however concentrations above these values may be considered harmful. The purpose of this study was to investigate the genotoxic effects of the pesticides monocrotophos, chlorpyriphos, dimethoate and endosulfan on human lymphocyte chromosomes.

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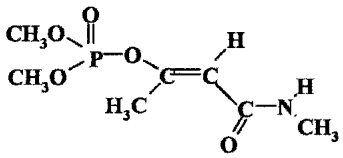
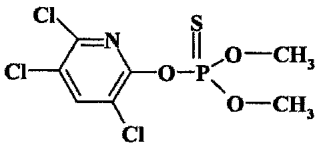
(i) Monocrotophos



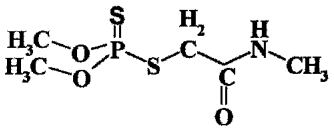
(ii) Chlorpyriphos



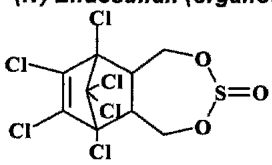
(i) Dimethyl (E)-1-methyl-2-methyl carbanoyl. Vinyl phosphate (IUPAC) C7H14NO5P (OP).

(ii) Di ethyl, 3, 5,6-trichloro-2-pyridyl phosphorothionate $C_9H_{11}Cl_3No_3PS$,

(iii) Dimethoate



(iv) Endosulfan (organochlorine)



(iii)O,O-dimethyl S-[2-methylamine)-2-oxoethyl] phosphorodithioate $C_5H_{12}NoPS_2$.

(iv)1,4,5,6,7,7-hexachloro-8,9,10-trinorborn-5-en-2,3-ylene-dimethylsulphite $C_9H_6Cl_6O_3S$.

Figure 1.Pesticides used in this study

The biological activity of chemicals has been studied either in-vitro or in-vivo, but following the recent trend in toxicological research most of the toxicologists have turned towards in-vitro methods as alternatives to in-vivo methods. Hence there is urgent need to determine the end-points of the in-vitro methods, in order to make the invitro methods widely applicable to xenobiotic agents. The aim of the present study was to utilize human lymphocyte cultures from peripheral blood samples for in-vitro studies. From practical as well as scientific point of view cultured mammalian cell lines, and peripheral blood lymphocyte cultures seem to be more suited for routine analysis for their predictive handling methods. The handling of human peripheral blood lymphocyte culture system is a simple system that provides us with a large number of dividing cells. This is particularly useful to detect the aberration frequencies in dividing cells.

MATERIALS AND METHODS

Organophosphorus pesticides (Technical grade) were obtained from Bhagirath Chemicals Ltd., Hyderabad, India and Endosulfan (Tech) was gift sample from Hindustan Insecticides Limited, Haryana, India (Fig1).

One percent stock solutions of all pesticides were prepared in DMSO (Dimethyl sulphoxide). Two concentrations of each pesticide (i.e.$1/10^{th}$ and close to $1/10^{th}$ of

LC_{50}) were used in the experiments to determine the chromosomal aberration frequencies. The LC_{50} values of these pesticides were as follows: 0.72±0.01mg/ml for Monocrotophos; 1.74±0.04mg/ml for Chlorpyriphos, 6.92±0.34 mg/ml for Dimethoate and 0.73±0.01mg/ml for Endosulfan (as reported earlier by Kaiser Jamil et al 2004). Venous blood samples drawn from healthy non-smoking donors were used for preparing lymphocyte cultures.

The procedure was essentially as described by Moorhead *et al* (1960). The culture media supplemented for each blood sample (1.5ml) consisted of 5ml of RPMI 1640 medium (5g/100ml) containing sodium bicarbonate (1g/100ml), fetal calf serum (10ml/100ml), penicillin (100IU/ml) and streptomycin (100IU/ml) (pH 7.2 -7.5). 2units of phytohemagglutinin (PHA) was added to each vial and incubated at 37^0C for 72 hrs. At the end of 48hrs, pesticide aliquots as mentioned above were added to the vials and incubated further for 24hrs at 37^0C.At 70^{th} hour colchicine solution (0.004 mg/ml, sigma) added arrest the cells at the metaphase stage. The lymphocytes were harvested after giving a hypotonic shock with KCl (0.56%) and then fixed in a 3:1; methanol: acetic acid solution. About 80-100 well spread metaphases for each treatment were evaluated for the presence of chromosomal aberrations after Geimsa staining. Whole blood without pesticide treatment was taken as positive control. As these pesticides were soluble in organic solvents like DMSO, this was taken as the negative control. All the experiments were performed in triplicates. The data were statistically analyzed using Pearson's correlation analysis.

RESULTS AND DISCUSSION

Cytogenetic analyses provide a valuable technique for evaluating the damage of pesticides to chromosomes (chromatids) on the basis of direct observation and classification of chromosomal aberrations. The aberrations were classified into categories like gaps, fragments, satellite associations; aneuploidy and dicentrics. Breaks were also recorded. Cells arrested in metaphase were examined for both numerical and structural aberrations. Different types of structural chromosomal aberrations are listed in the table with their numbers and frequencies.

Results in human cell models give some insight into the different mechanism involved in cellular toxicity. Also it is generally believed that physical, chemical and structural properties of a pesticide are the indicators of their toxic nature. This principle forms the basis for the prediction of toxicity from chemical structure. The lymphocyte cultures from peripheral blood are inexpensive, simple to perform reliable, repeatable and therefore suitable material for routine analysis. Stimulated lymphocytes enter metaphase cycle within 48 hours in vitro thus permitting analysis of their first division. The immune response of lymphocytes can also be tested with unspecific mitogens. Mixed cultures of lymphocytes from different donors may be used as in vitro methods for histo-compatability. The analysis of chromosomes from nucleated cells of the hemopoietic system particularly lymphocytes, has become the main method in cytogenetics, the effects of toxic substances on cells and chromosomes in-vitro can be relatively simple to test (Hagmar etal 2001).

Figure 2: Normal male Karyotype showing 22 pairs of chromosomes and one pair of sex chromosomes (46 chromosomes).

Since the purpose of this investigation has been to analyze the spectra of chromosome aberrations induced by low doses of pesticides, the karyotype of normal metaphase is presented in figure-2 and the metaphase plates of treated samples is presented in figure3 (a & b).

Our findings on chromosomal aberrations using pesticides like Monocrotophos, Chlorpyriphos, Endosulfan and Dimethoate have shown various types of aberration, which are dose, dependent. As the concentration of the pesticides increases there was more number of aberrations (shown in the Table). An advantage is that stable rearrangements persist and thus provide an integrated record of exposure.

Analysis of the results as presented in the Table shows the total spectrum of frequencies in chromosome aberrations. Monocrotophos induced chromosomal aberrations in the metaphase plates such as chromatid gap, dicentric (A-1and B-4) and satellite association between D and D. Chlorpyriphos induced satellite association between D and G group of chromosomes and chromatic gap was also recorded. Endosulfan treatment showed satellite associations between D and G group of chromosomes, fragment, and chromatid break were also observed. Dimethoate induced satellite association seen between D and D, D and G group of chromosomes, and fragment and gap were also observed.

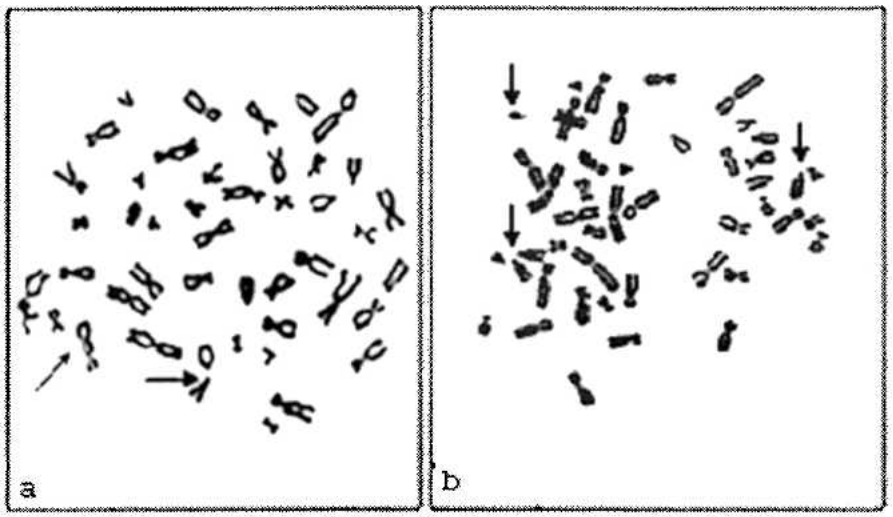


Figure3): Metaphases showing aberrations after the treatment with pesticides.
a) Showing dicentric and satellite association. b) Showing fragment, satellite association.

Table Analysis of human lymphocyte chromosomes treated with pesticides

	Control	Monocrotophos µM		Chlorpyrephos µM		Endosulfan µM		Dimethoate µM	
		8	16	28	56	14	16	43	86
Cells counted	100	100		100		100		100	
Breaks	-	5±1.00	8±1.15	-	2±0.57	-	-	6±1.0	7±1.5
Gaps	-	5±1.00	10±1.15	6±1.15	8±1.00	1±0.5	2±1.00	7±0.5	10±2.0
Fragments	-	1±0.57	2±1.00	-	4±0.57	-	1±0.57	-	2±0.57
Satellite associations	6 ± 0.57	20 ± 0.57	15 ± 1.73	15 ± 2.00	19 ± 1.00	13 ± 1	10 ± 0.57	15 ± 2.00	14±1.15
Aneuploidy	-	4±1.00	6 ± 1.15	9 ± 0.57	12 ± 0.57	7 ± 1.15	9 ± 0.57	9 ± 0.57	12 ± 0.57
Dicentrics	-	6±2.00	8±1.00	7±1.00	8±1.00	-	1±1.00	5±2.0	8±1.7
Total aberrations	2	41	49	37	53	21	23	39	53

*$p<0.05$ number ± standard error

The formation of satellite association has often been attributed to the involvement of satellite in nuclear formation. The sticky molecular material would have a tendency to hold the associated chromosomes together through mitosis. A high

incidence of satellite associations has often been considered as predisposing to an increased tendency of nondisjuction in satellite chromosome and thus leads to the induction of D & G trisomies. Our results also show that the frequency of D & G associations was more. These results indicate the non-random involvement of acrocentrics compared to controls. Thus the frequency of satellite association in our investigation could be due to predisposition to non-disjunction and the results seem consistent with the hypothesis that acrocentric associations are related with non-random distribution of acrocentric non-disjunction.

In this study we have observed dicentric aberrations more often in the A group of chromosomes and sometimes in other larger chromosomes. Brogger and Johansen (1972) have reported that it is possible to enhance the frequency of secondary constriction in the chromosome by certain chemicals like 5-bromodeoxy uridine (BudR), hydroxylamine hydrochloride or mitomycin. This is the first report of pesticide related chromosomal aberrations where more dicentrics breaks and gaps have been reported. With increasing gradient of the pesticide the percentage of dicentric and satellite associations also increased. Among the metaphases examined so far, we observed the frequency of autosomal chromosomal aberration was higher. Knowledge of this type of variation in polymorphism is important in differentiating them from normal to pathological conditions and to determine the precise location of the chromosomal aberration frequency in chromosomal rearrangements. Chromosome breaks may represent damaged regions that have not undergone repair. It is common to find satellite regions of the acrocentric chromosomes of the D & D and D & G groups in some normal karyotype (Jeno major1999; Hansson A 1970). But there was a significant increase in satellite associations in pesticide treated samples.

The distinction between the chromatid gap and break is very arbitrary. A gap cytogenetically is distinguished from a break depending on the length of the non-staining region in the chromosome. If the attenuated region is shorter than the width of the chromatid, it has been classified as a gap and if longer than the width it is a break. It is seen from our results that under the influence of pesticides, the frequencies of both gaps and breaks has increased, it has been suggested by many authors that gaps are indicative of toxic phenomena from genetic point of view, and these aberrations could be useful sensitive biomarkers of pesticide induced genetic damage.

Overall, low dose of pesticides appear to have the capability to alter the genetic material particularly chromosomes in mammalian cultures (Bolognesi 2003;Antonucci 2000) Chromosomal aberrations would seem to meet many of the requirements of a good test system. However, the scoring for chromosomal aberrations has to be done by an expert, which limits use for wider applications, as it is time consuming. Perhaps no test system has been as extensively studied and applied as the structural and numerical changes that can be observed in circulating lymphocytes. At moderate exposures, one still must assign increased risk to a population rather than to individuals, although the required population may be of modest size. An advantage is that stable rearrangements persist and thus provide an integrated record of exposure.

Cells exposed to pesticides undergo DNA breakage and misrejoining. Low exposures during G0/G1 phase of cell cycle produce primary chromosome aberrations indicating genomic instability. These form important tools in quantitative analysis showing whether data indicate some specific deviation from controls. Genomic instability could be important in inducing the development of cancers.

Biomarkers for pesticide toxicology are important parameters, which have been determined in this investigation. . It is also known that stable marker chromosome seemed to be derived from chromosomes of group "A" and "B" One common feature of pesticide genotoxicity was the frequency of dicentrics and satellite associations, which was of much interest and is being reported for the first time as biomarkers of pesticide toxicity. Frequency of chromosomal aberrations from nucleated cells of hemopoietic system particularly lymphocytes have proved to be an invaluable method for in-vitro studies. Moreover the in-vitro chromosomal aberration test may employ cultures of established cell lines, cell strains, or primary cell cultures.

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