Effect of paraoxon-methyl and parathion-methyl on DNA in human lymphocytes and protective action of vitamin C

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Abstract: The molecular basis of the genotoxicity of the commonly used organophosphorus insecticide, parathion-methyl is poorly understood and there is a lack of information on the possible effects of its metabolic conversion products. In the present work the action of parathion-methyl and its immediate metabolite paraoxon-methyl on DNA in human lymphocytes was compared using the comet assay. Parathion-methyl at 25 and 75 μM did not cause any significant changes but at 200 μM a significant increase in the tail moment was observed as compared with the control. Paraoxon-methyl at 25, 75 and 200 μM evoked dose-dependent DNA damage measured as a significant increase in comet tail moment of lymphocytes. The change evoked by paraoxon-methyl at 200 μM was much more pronounced than that by parathion-methyl at the same concentration. To search for the mechanism underlying the observed effect, the action of a well-known antioxidant, vitamin C, along with parathion-methyl and paraoxon-methyl was studied. The vitamin at 10 and 50 μM reduced the DNA-damaging activity of paraoxon-methyl at all its concentrations. The results indicate that the reported genotoxic effects of parathion-methyl could be mainly attributed to its metabolite paraoxon-methyl. The protective action of vitamin C suggests that paraoxon-methyl may cause oxidative DNA damage.

Keywords: parathion-methyl; paraoxon-methyl; DNA damage; vitamin C; comet assay

1 INTRODUCTION

Pesticides can affect man, domestic and wild animals. Most pesticides do not show any immediate adverse effect (at least at concentrations normally used) but may pose a significant long-term hazard to man.[1] Genotoxicity of pesticides is of special significance because of the common presence of these agents in the environment and the long latent period between the exposure and effects becoming apparent.[2]

Parathion-methyl is a widely used organophosphorus insecticide.[3] The acute toxic action of this compound is due to the inhibition of acetylcholinesterase of the nerve tissue, following its metabolic conversion to the oxygen analogue paraoxon-methyl,[4] (Fig 1). There is some evidence for the genotoxic activity of parathion-methyl, as reported with short-term bacterial tests,[5–7] but there is little information on the genotoxicity of its metabolites, although it is well known that many chemical carcinogens require metabolic activation to exert their biological effect. In addition, the molecular mechanism(s) of the observed mutagenic changes has not yet been elicited.

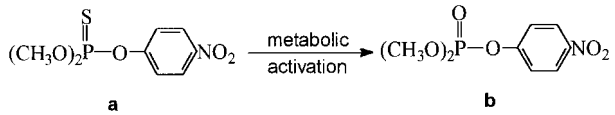

Figure 1. Chemical structures of (a) parathion-methyl and (b) paraoxon-methyl.

Vitamin C (ascorbic acid) is a water-soluble dietary antioxidant that plays an important role in controlling the oxidative stress.[8] It can protect DNA against damage induced by reactive oxygen species.[9] Vitamin C is also a powerful reductant and may react with copper or iron in the cell which produce free radicals.[10–12] These properties reflect a dual, anti- and pro-oxidative, nature of vitamin C and it is important to establish a mode of its action in the presence of a specific substance under particular cellular conditions.[13]

DNA damage induced by chemicals appears primarily in the form of alterations to the phosphate backbone, sugar or base modifications such as alkylations, cross-links, or formation of bulky DNA adducts, which are substrates for DNA repair mechanisms. Transient DNA breaks arise in the second step as a consequence of repair and can be considered as important markers of genotoxicity.[14] Single cell gel electrophoresis (comet assay) is a sensitive genotoxi-

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city test to investigate DNA damage and repair.[14–16] In this technique a small number of cells suspended in a thin agarose gel on a microscope slide are lysed, electrophoresed and stained with a fluorescent DNA-binding dye. The principle of the method is that broken DNA molecules can migrate more readily in an electric field than intact molecules. When cells are embedded in agarose and subsequently lysed to remove proteins, smaller DNA fragments are able to migrate away from the residual nucleus. After subsequent DNA staining with a fluorescent DNA-binding dye and visualisation using a fluorescence microscope, the observed objects resemble comets with a head region containing undamaged DNA and a tail containing the broken DNA. The amount of DNA able to migrate and, to a lesser extent, the distance of migration, are indications of the number of strand breaks present in that cell. Cells with an increased level of DNA damage display an increased migration of chromosomal DNA from the nucleus towards the anode. In the alkaline version of the comet assay, DNA single strand breaks and alkali-labile sites become apparent, and the extent of DNA migration indicates the level of DNA breakage in the cell.[14] It has been shown that the comet assay can detect a broad spectrum of mutagens.[17]

In the present work the ability of parathion-methyl and paraoxon-methyl to induce DNA damage in human lymphocytes was investigated using the comet assay. Additionally, a potential modulating effect of vitamin C on the action of these two compounds was tested.

2 MATERIALS AND METHODS

2.1 Chemicals

Parathion-methyl and paraoxon-methyl at purity of 99.8% were supplied by Dr Ehrenstorfer GmbH (Augsburg, Germany). RPMI 1640 medium without glutamine, low melting point agarose, buffered phosphate saline (PBS), L-ascorbic acid, sodium salt and DAPI (4′,6-diamidino-2-phenylindole) were obtained from Sigma (St Louis, MO, USA). Gradisol L was from Polfa (Kutno, Poland). All other chemicals were of analytical grade and were purchased from Sigma (St Louis, MO, USA).

2.2 Lymphocyte isolation

Blood was obtained from young, healthy, non-smoking donors. Peripheral blood lymphocytes (PBL) were isolated by centrifugation in a density gradient of Gradisol L (15 min, 280 *g*).[18] The viability of the cells was measured by Trypan blue exclusion and found to be about 99%. Lymphocytes accounted for about 92% of leukocytes in the cell suspension produced. The final concentration of the lymphocytes was adjusted to $1–3 \times 10^5$ cells ml^{-1} by adding RPMI 1640 medium to the single cell suspension.

2.3 Chemical treatment

Parathion-methyl and paraoxon-methyl were taken from stock (50 mM) ethanolic solutions and added to the suspension of lymphocytes to give final concentrations of 25, 75 and 200 μM. The concentration of the chemicals in the working solutions was checked spectrophotometrically by comparing their absorption spectra with the spectra of parathion-methyl and paraoxon-methyl dissolved in pure ethanol, with a correction for solvent absorption. The control cells were treated with ethanol alone at a concentration of 0.18%, which did not affect the processes under study (data not shown). To examine DNA damage, the lymphocytes were incubated with the chemicals for 1 h at 37 °C. Each experiment included a positive control, which was treated with 20 μM H_2O_2, producing pronounced DNA damage resulting in a comet length of 80–120 μm (results not shown). In experiments with vitamin C, incubation with organophosphates was preceded by addition of 10 mM sodium ascorbate to the suspension of the lymphocytes to give final concentrations of 10 or 50 μM.

2.4 Comet assay

The comet assay was performed under alkaline conditions using a slight modification of the procedure of Singh *et al.*[15] A freshly prepared suspension of lymphocytes in 0.75% low melting point agarose dissolved in PBS was cast onto fully frosted microscope slides (Superior, Germany) precoated with 0.5% normal melting agarose. The cells were then lysed for 1 h at 4 °C in a buffer consisting of sodium chloride (2.5 M), EDTA (100 mM), Triton X-100 (10 g litre^{-1}) and Tris (10 mM; pH 10). After the lysis the slides were placed in an electrophoresis unit, allowing DNA to unwind for 40 min in the electrophoretic buffer consisting of sodium hydroxide (300 mM) and EDTA (1 mM; pH > 13). Electrophoresis was conducted at an ambient temperature of 4 °C (the temperature of the running buffer not exceeding 12 °C) for 30 min at an electric field strength 0.73 V cm^{-1} (30 mA). The slides were then neutralised with Tris, (0.4 M; pH 7.5), stained with DAPI (2 μg ml^{-1}) and covered with cover slips. To prevent additional damage all the steps described above were conducted under a dimmed light or in the dark.

2.5 Comet analysis

The objects were observed at 200 × magnification in a Labophot-2 fluorescence microscope (Nikon, Japan) attached to a Pulnix video camera (Kinetic Imaging, Liverpool, UK) equipped with a UV-1A filter block (an excitation filter of 365/10 nm and a barrier filter of 435 nm) and connected to a personal-computer-based image analysis system Comet v 3.0 (Kinetic Imaging, Liverpool, UK). Fifty images were randomly selected from each sample and the comet tail moment (a product of fraction of DNA in tail and tail length) was measured. Two parallel tests with aliquots of the same sample of cells were performed for a total of 100 cells

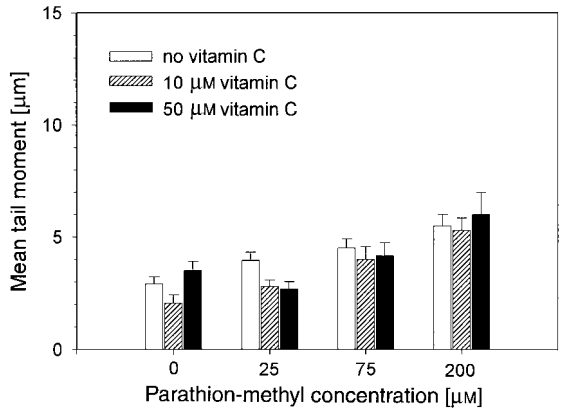

Figure 2. Mean comet tail moment of human lymphocytes exposed for 1 h at 37 °C to parathion-methyl in the presence and absence of vitamin C. The number of cells in each treatment was 100. Error bars denote SEM.

and the mean comet length was calculated. The comet tail moment is positively correlated with the level of DNA breakage in a cell.[15] Because the distribution of the comets was heterogeneous, histograms were used to display information. The mean value of the tail moment in a particular sample was taken as an index of DNA damage in this sample.

2.6 Statistics

All the values in this study were expressed as mean (±SEM) from two separate experiments. The data were analysed using STATISTICA (StatSoft, Tulsa, OK, USA) statistical package. To detect any significant changes between groups, one-way analysis of variance was used. The differences between means were compared using Scheffe's multiple comparison test.[19]

3 RESULTS

The mean comet tail moments for the lymphocytes exposed for 1 h to parathion-methyl with and without vitamin C were compared with appropriate controls (Fig 2). Parathion-methyl at 25 and 75 μM did not evoke any significant change in the tail moment of lymphocytes but at 200 μM a significant increase in the tail moment was observed as compared with the control (5.50 (±0.51) μm vs 2.91 (±0.31) μm, $P < 0.01$). Vitamin C did not exert any significant effect on the tail moment of the lymphocytes at either tested concentration.

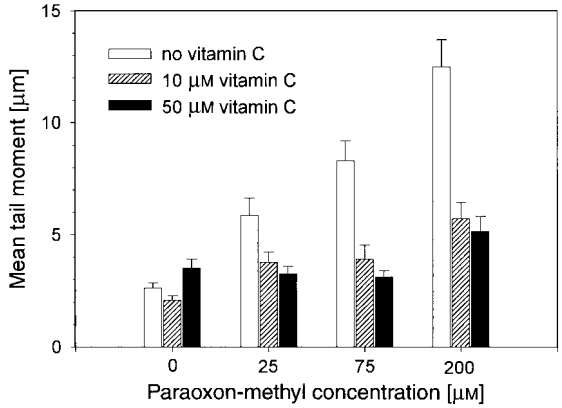

Figure 3. Mean comet tail moment of human lymphocytes exposed for 1 h at 37 °C to paraoxon-methyl in the presence and absence of vitamin C. The number of cells in each treatment was 100. Error bars denote SEM.

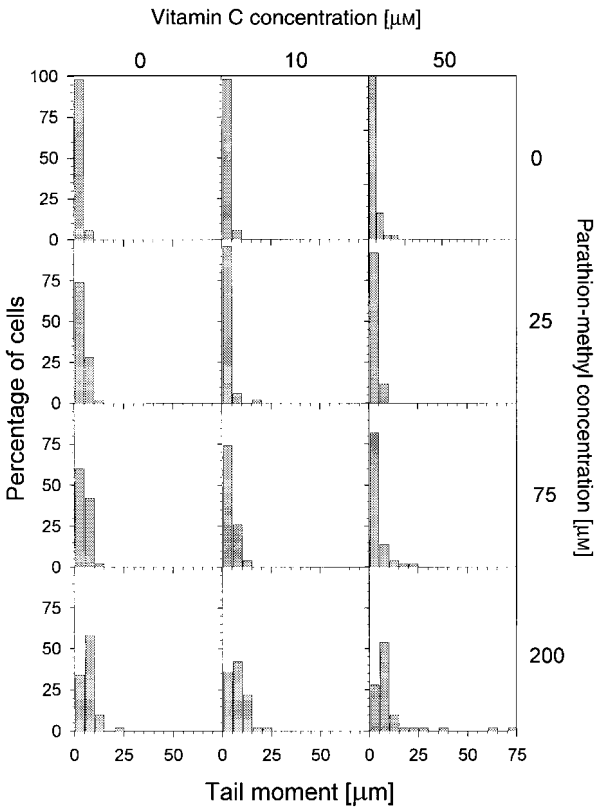

Figure 4. Histograms of the distribution of comet tail moment in human lymphocytes treated for 1 h at 37 ° with parathion-methyl in the presence and in the absence of vitamin C. The number of cells scored for each treatment was 100.

Figure 3 shows the mean comet tail moments for the lymphocytes exposed for 1 h to paraoxon-methyl in the presence or absence of vitamin C. It can be seen from the figure that paraoxon-methyl significantly increased the tail moment of the lymphocytes in a dose-dependent manner. At the highest concentration of the chemical, 200 μM, the increase of the tail moment was over four times the initial value (2.61 (±0.22) μm vs. 12.48 (±1.22) μm, $P < 0.001$). Vitamin C decreased the tail moment of the lymphocytes exposed to paraoxon-methyl. The effect was concentration-independent and vitamin C decreased the tail moment to its initial value at paraoxon-methyl concentrations of 25 and 75 μM; at 200 μM the decrease caused by vitamin C exceeded 50% ($P < 0.001$).

The most basic way of viewing the data from the comet assay is the distribution of cells according to the percentage of DNA in the tail, which is positively correlated with the tail moment.[20] Figures 4 and 5 show the distribution of lymphocytes according to their comet tail moments after treatment with parathion-methyl and paraoxon-methyl, respectively. It can be seen from Figure 4 that increasing the concentration of paraoxon-methyl caused an increase

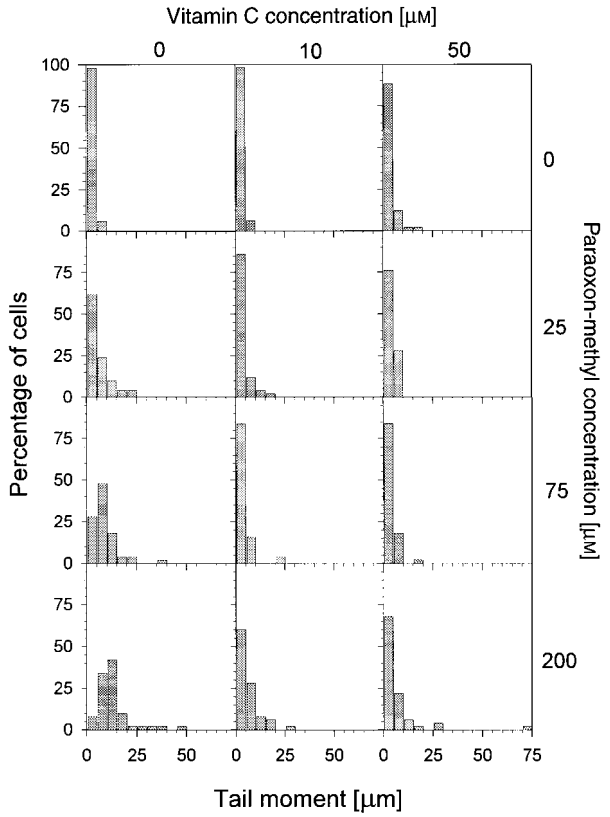

Figure 5. Histograms of the distribution of comet tail moment in human lymphocytes treated for 1 h at 37° with paraoxon-methyl in the presence and in the absence of vitamin C. The number of cells scored for each treatment was 100.

in the fraction of lymphocytes with greater comet tail moments compared with the unexposed control. These comets contained more DNA in their tails, which indicated greater DNA damage in the treated cells. The action of vitamin C at both 10 and 50 μM apparently reduced the fraction of lymphocytes with greater tail moment. Parathion-methyl at 25 and 75 μM did not alter significantly the distribution of the lymphocytes due to their percentage of DNA contents in the comet tail but at 200 μM it increased the population of lymphocytes with greater tail moments.

4 DISCUSSION

The concentrations of the tested organophosphorus compounds in the study ranged from 25 to 200 μM, which corresponds to about 8–66 μg ml^{-1}. It was estimated that in extreme cases agricultural workers could be occupationally exposed to doses of around 10 μg ml^{-1} of organophosphates,[21] which is of the same order as the lowest exposure value in the present experiment but much higher than the actual concentration in the blood because of the relatively rapid metabolism in relation to the rate of absorption.

The results obtained indicate that parathion-methyl and its metabolically activated form paraoxon-methyl have an ability to damage DNA in human lymphocytes. The effect of parathion-methyl was observed only at the highest concentration, 200 μM, whereas paraoxon-methyl damaged DNA at all concentrations tested. The effect evoked by paraoxon-methyl at 200 μM was much more pronounced than that of its parent compound. Vitamin C at concentrations in the human physiological range protected lymphocyte DNA against damage caused by paraoxon-methyl. At concentrations of the latter of 25 and 75 μM, the protection was complete; at 200 μM, the extent of DNA damage was diminished to about half of its initial value.

Approximately 80% of the total metabolism of parathion-methyl in the mouse and rat proceeds in the liver by the microsomal mixed function oxidase system.[22] The possibility of the metabolism of parathion-methyl by enzymes present in the human lymphocyte microsomal fraction cannot be excluded, although the effectiveness of this process of metabolic transformation in the lymphocyte is expected to be low compared to that in the liver. The relative levels of the effects of parathion-methyl and paraoxon-methyl could be considered as a measure of the part of parathion-methyl that underwent metabolic transformation to paraoxon-methyl in lymphocytes.

Parathion-methyl at 25 and 75 μM did not show an ability to damage DNA in the experiment, although it is considered to be genotoxic as measured with bacterial tests.[5–7,23–25] Parathion-methyl was also shown to interact directly with isolated DNA, changing its thermodynamic properties as assessed by thermal denaturation and circular dichroism studies.[26] In that experiment parathion-methyl was not able to undergo any metabolic transformation. Parathion-methyl can also covalently bind to isolated DNA.[26] Taking these facts together, one can conclude that parathion-methyl has an ability to interact directly with DNA, but the results of our experiments indicate that this interaction is not necessarily a basis of reported genotoxic properties of this compound. Instead, parathion-methyl could first undergo metabolic activation to its oxon form which could then damage DNA and, in consequence, produce a genotoxic effect.

As underlined in the Introduction, genotoxicity of pesticides is of special significance. Although 'genotoxicity' is a useful term whose precise definition is elusive, there is no doubt that DNA damage plays a pivotal role in most mechanisms underlying genotoxicity.[27] Therefore, the ability of paraoxon-methyl to generate DNA breaks can lead to genotoxic effects, but the experiment described here did not prove that paraoxon-methyl is genotoxic *in vivo*. It did prove, however, that the compound has a potential to interact directly with DNA and it is therefore worth investigating further.

Both organophosphorus compounds were dissolved in ethanol, which can have an antioxidant activity.[28] We observed (data not presented), that ethanol at the concentration applied in our study (0.18%) slightly, but statistically significantly, decreased DNA damage induced by hydrogen peroxide. However, it should be taken into account, that ethanol was present in all

modes of exposure, ie with and without vitamin C as well as in the controls.

Vitamin C has emerged from a wide variety of research as an antioxidant of major importance against a variety of oxidative stress conditions.[29] Besides exerting antioxidant influence *per se*, vitamin C can promote the removal of oxidative DNA damage from the DNA and/or nucleotide pool, via the upgrading of repair enzymes, perhaps induced by vitamin C's pro-oxidant properties.[30,31] It should be taken into account that normal plasma concentrations of vitamin C ranged from 34 to 114 μM,[32] but much higher concentrations are found in leukocytes, including lymphocytes, which concentrate vitamin C to concentrations 14-fold greater than those in plasma.[33]

Taking all the evidence together one can conclude that: (1) genotoxic effects of parathion-methyl may be caused by oxidative DNA damage evoked by its immediate transformation product paraoxon-methyl and (2) vitamin C exerts a protective action against DNA damage caused by paraoxon-methyl.

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